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(54) Polyethylene glycol protein conjugates.

(57) The present invention relates to physiologically active, substantially non-immunogenic water soluble polyethylene glycol protein conjugates having at least part of the biological activity of the protein which forms the conjugate, without exhibiting the same disadvantageous immunogenic responses thereof.

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The present invention relates to novel polyethylen glycol (PEG) conjugates of proteins.

Various natural and recombinant proteins have medical and pharmaceutical utility. Once they have been purified, separated, and formulated, they can be parenterally administered for various therapeutic indications. However, parenterally administered proteins may be immunogenic, may be relatively water insoluble, and may have a short pharmacological half life. Consequently, it can be difficult to achieve therapeutically useful blood levels of the proteins in patients.

These problems may be overcome by conjugating the proteins to polymers such as polyethylene glycol. Davis et al., U.S. Pat. No. 4,179,337 disclose conjugating polyethylene glycol (PEG) to proteins such as enzymes and insulin in order to result in conjugates where the protein would be less immunogenic and would retain a substantial proportion of its physiological activity. Nakagawa, et al., U.S. Pat. No. 4,791,192 disclose conjugating PEG to islet-activating protein to reduce its side-effects and immunogenicity. Veronese et al., Applied Biochem. and Biotech, 11:141-152 (1985) disclose activating polyethylene glycols with phenyl chloroformates to modify a ribonuclease and a superoxide dimutase. Katre et al. U.S. Pat. Nos. 4,766,106 and 4,917,888 also disclose solubilizing proteins by polymer conjugation. PEG and other polymers are conjugated to recombinant proteins to reduce immunogenicity and increase half-life. See Nitecki, et al., U.S. Pat. No. 4,902,502, Enzon, Inc., International Application No. PCT/US90/02133, Nishimura et al., European Patent Application 154,316 and Tomasi, International Application Number PCT/US85/02572. King et al., Int. Archs. Allergy appl. Immun. 66, 439-446 (1981) describes a method for linking proteins to PEG by using O-(RO-PEG)-S-carboxamidomethyl-dithiocarbonate intermediates.

Previous methods of forming PEG-protein conjugates and the conjugates which result from said methods present several problems. Among these problems is that certain methods of forming these protein-PEG conjugates may inactivate the protein so that the resulting conjugates may have poor biological activity. In addition, certain linkers utilized in forming these PEG-protein conjugates may be susceptible to in vivo hydrolytic cleavage. When such cleavage occurs after administration, these conjugates lose the beneficial properties provided by PEG.

The present invention provides novel PEG-protein conjugates through the use of unique linkers which connect the various free amino groups in a protein to PEG which circumvent the problems associated in the formation of conjugates of a protein with PEG.

In particular the present invention provides a physiologically active conjugate of a protein of the formula

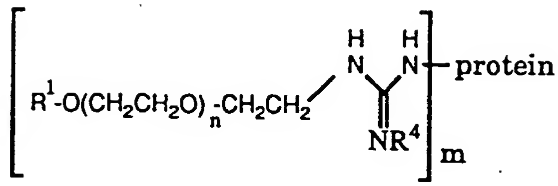
$$\left[R^1\text{-O-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}R^2\text{-}\underset{\underset{\displaystyle R^3}{\|}}{C}\text{-NH} \right]_{m}\text{-protein} \qquad \mathrm{I}$$

wherein R^1 is lower alkyl; R^2 is -O- or -NH-; R^3 is $=N\text{-}R_4$, $=S$ or $=O$; R^4 is lower alkyl or cycloalkyl; m and n are selected from integers ≥ 1 in a way that the conjugate has at least a portion of the biological activity of the non conjugated protein;

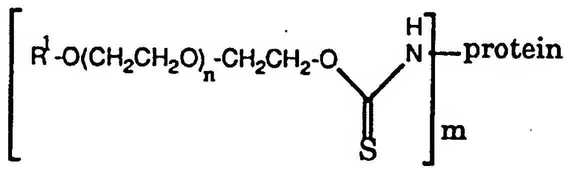
with the proviso that when R^2 is -O-, R^3 is other than $=N\text{-}R^4$;

that when R^2 is -O- and R^3 is $=O$ the protein is interferon-alpha, interleukin-1 or interleukin-1ra and that when R^2 is -O- and R^3 is $=S$ the protein is not antigen E from ragweed pollen or bovine plasma albumin.

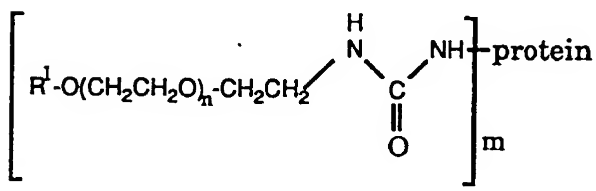
More specifically, the present invention provides protein conjugates of the formulas:



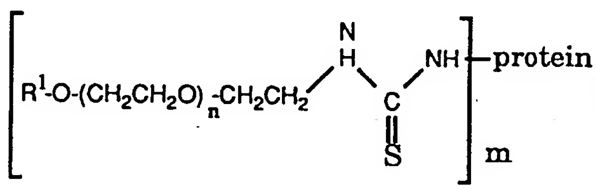
I-A



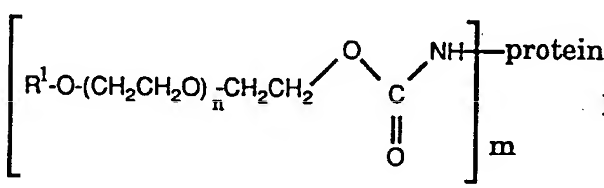
I-B



I-C



I-D



I-E

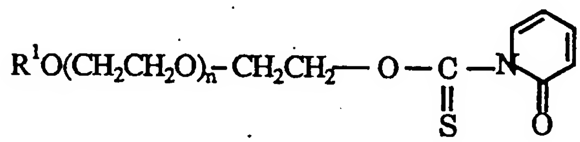
wherein R^1, R^4, m, n and the protein are as above.

In accordance with the present invention, we have also provided for the first time the protein interleukin-1 receptor antagonist (IL-1ra) and interleukin-1 (IL-1) conjugated to PEG.

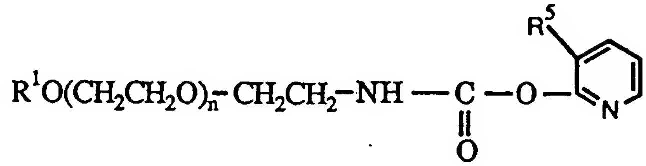
In accordance with this invention, the conjugate of a protein of formula I is produced by condensing activated PEG, viz. PEG wherein one hydroxy group has been replaced by an activated linker, with one or more of the free amino groups in the protein. The activated PEG compounds used to produce the conjugate have the following formulas:

II-A $R^1O(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}N{=}C{=}N\text{-}R^4$

II-B

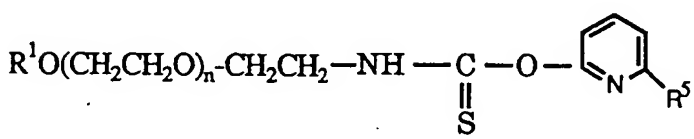


II-C

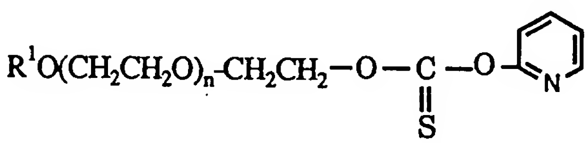


II-D $R^1O(CH_2CH_2O)_n\text{-}CH_2\text{-}CH_2\text{-}N{=}C{=}S$

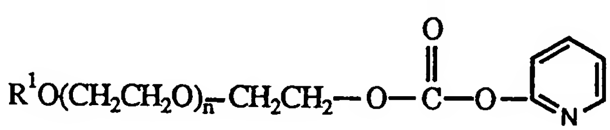
II-E



II-F



II-G



wherein R^1 is lower alkyl, R^4 is lower alkyl or cycloalkyl, and R^5 is lower alkyl or H and n is an integer of from 1 to 1000.

In accordance with this invention, by using the activated PEG compound of formula II-A, II-B, II-C, II-D, II-E, II-F or II-G to produce the conjugates, a linking bond between the free amino groups in the protein and the PEG is formed so that the resulting conjugate retains at least a portion of the biological activity of the protein while reducing its immunogenicity. In addition, the linkage groups formed in the conjugate of this invention through the use of any one of the activated polyethylene glycols of formulae II-A through II-G produces a protein conjugate which is not readily susceptible to in vivo hydrolytic cleavage and is not subject to many of the disadvantages present in the PEG protein conjugates of the prior art.

In accordance with this invention, R^1 and R^5 can be any lower alkyl, preferably methyl. The term lower alkyl designates lower alkyl groups containing from 1 through 6 carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, etc. Generally the preferred alkyl group is a lower alkyl group containing from 1 to 4 carbon atoms with methyl being most preferable.

In accordance with this invention, m and n can be selected from any integer ≥ 1 in a way such that the resulting conjugate of a protein has at least a portion of the biological activity of the protein which forms the conjugate. It is apparent that the sum of n and m is inversely proportional to the amount of biological activity of the protein which is retained by the conjugate. The numerical value of n relates the number of ethylene glycol units in the polyethylene glycol which form the conjugate and is between 1 and 1000. The term m relates to the number of free amino groups contained by the protein which is reacted with the activated PEG. Preferably the value of m is in the range of 1 to 5 with 1 being the most preferred one. The higher the value of m + n, the higher is the molecular weight of the conjugate. The molecular weights of the polyethylene glycol polymers of formulae II-A to II-G and of the protein conjugates of formulae I and I-A to I-E can be easily determined by a person skilled in the art. From the molecular weights determined the

values of m and n can be deduced. The term protein comprises on the one hand polypeptides and on the other hand physiologically acceptable addition salts.

When the compound of any one of formula II-A through II-G is reacted with the protein and the protein contains more than one free amino group, the conjugate may be produced as a mixture of various protein PEG conjugates. In cases where the protein contains two free amino groups, the activated PEG can react both with one of the free amino groups and with both of the free amino groups. In this situation the mixture contains one conjugate formed where two free amino groups are reacted with PEG and a second conjugate formed where only one free amino group is reacted with PEG. Since the various conjugates in this mixture have different molecular weights, these conjugates can be separated by conventional methods such as chromatography. To determine if m and n have been selected properly, the separated conjugates can be screened for biological activity by the same means used to screen the parent protein to determine if the conjugate still retains a portion of the biological activity of the protein used to form the conjugate. In this manner, the numbers m and n can be adjusted in any desired manner to provide the desired activity.

In accordance with the preferred embodiment of this invention m and n are any integer so that molecular weight of the conjugate, excluding the weight of the protein, is between approximately 300 to approximately 30,000 daltons. In accordance with the preferred embodiment, m is 1. Where m is 1, this conjugate can be obtained even when there are two or more free amino groups. The activated PEG compound will react first with one of the free amino groups contained within the protein groups. By regulating the concentration of the reagents such as the protein, and reaction conditions, in accordance with standard methods of amine condensation, one can regulate the degree of pegylation of the free amino groups contained within the protein. In proteins containing one or more free amino groups, where one of the free amino groups is more reactive than the other amino groups, conditions may be selected so that the protein is reacted with the activated PEG compound to form the compound of formula I where m is 1. Other free amino groups contained within amino acids which form the protein may be subsequently reacted with the PEG by allowing the condensation reaction to proceed longer or by utilizing other stronger conditions. In accordance with a preferred embodiment where m is 1, n is any integer so that the polyethylene glycol which forms the conjugate has a molecular weight of from 300 to 30,000 daltons corresponding to n in being approximately 6 to 680. More preferably n is about 28, 112 and 225 corresponding to molecular weights of 1325, 5000 and 10000 respectively, with n being in the region of 112 as the most preferred one. Characterizing the polyethylene glycol polymer by molecular weight is preferred over designating the self repeating units in the PEG polymer with the integer n due to the potential inhomogeneity of the starting PEG compounds which are usually defined by their avarage molecular weight and not their self-repeating units. The starting PEG compounds of various molecular weights can be prepared by methods known in the art or can be obtained from commercial suppliers.

In case the values of m and n obtained by determination of the molecular weights are not integers (as will generally be the case), there values have to be rounded up or off in the usual way.

Where R^4 is lower alkyl, R^4 can be any lower alkyl group containing from 1 to 6 carbon atoms such as defined above. When R^5 is cycloalkyl, R^5 is preferably a cycloalkyl group containing from 3 to 7 carbon atoms such as cyclopropyl, cyclopentyl, cyclobutyl, and cyclohexyl. The preferred cycloalkyl group is cyclohexyl.

The title compounds of each of the following Examples are named in accordance with IUPAC nomenclature. However, these compounds may also be named as follows:

Examples 1, 2, 3, and 4:

Alpha-[2-[[cyclohexylcarbonimidoyl)-amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-[2-[[(cyclohexylamino)methylene]amino]ethyl]omega-methoxypoly-(oxy-1,2-ethanediyl).

Examples 1A, 1a, and 1d:

Alpha-(2-chloroethyl)-omega-methoxypoly(oxy 1,2-ethanediyl) is alternatively named alpha-methoxy-omega-(2-chloroethyl)poly(oxy-1,2-ethanediyl).

Examples 1B, 1b, and 1e:

Alpha-(2-azidoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-methoxy-omega-(2-azidoethyl)poly(oxy-1,2-ethanediyl).

Examples 1C, 1c, and 1f:

Alpha-(2-aminoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-methoxy-omega-(2-aminoethyl)poly(oxy-1,2-ethanediyl).

Examples 11, 11a, 11b, 12, 12A, and 13-17: Alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]-ethoxy]poly(oxy-1,2-ethanediyl) is alternatively named alpha-[2-[[(2-pyridinyloxy)carbonyl]amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl).

Examples 18, 18a, 19, 19A, 20, 20A, 21, and 22:

Alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-methyl-omega[2-[[(2-pyridinyloxy)carbonyl]oxy]-ethoxy]poly(oxy-1,2-ethanediyl).

Examples 23-27:

Alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-[methoxy-omega-[2-(isothiocyanato)ethyl]poly(oxy-1,2-ethanediyl).

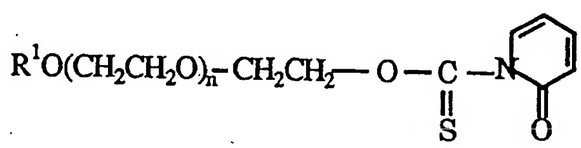
Example 28:

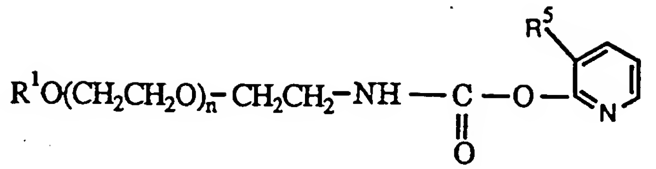
Alpha-[(2-pyridinyloxy)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) is alternatively named alpha-methyl-omega-[2-[[(2-pyridinyloxy)thiocarbonyl]oxy]ethoxy]-poly(oxy-1,2-ethanediyl).

The present invention also comprises the process for the preparation of the PEG-protein conjugates of the general formula I or formulae I-A through I-E which process comprises reacting one of the activated compounds of the general formulae

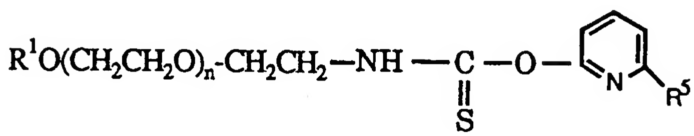
$$\textbf{II-A}\quad R^1O(CH_2CH_2O)_n\text{-}CH_2CH_2-N=C=N-R^4$$

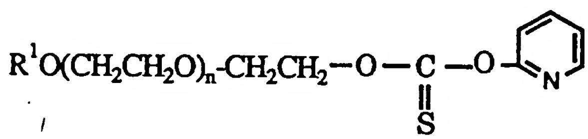
II-B

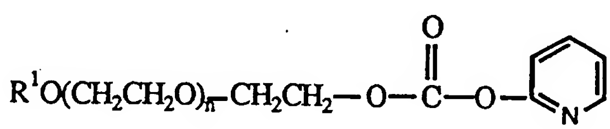


II-C 

II-D $R^1O(CH_2CH_2O)_n\text{-}CH_2\text{-}CH_2\text{—}N=C=S$

II-E 

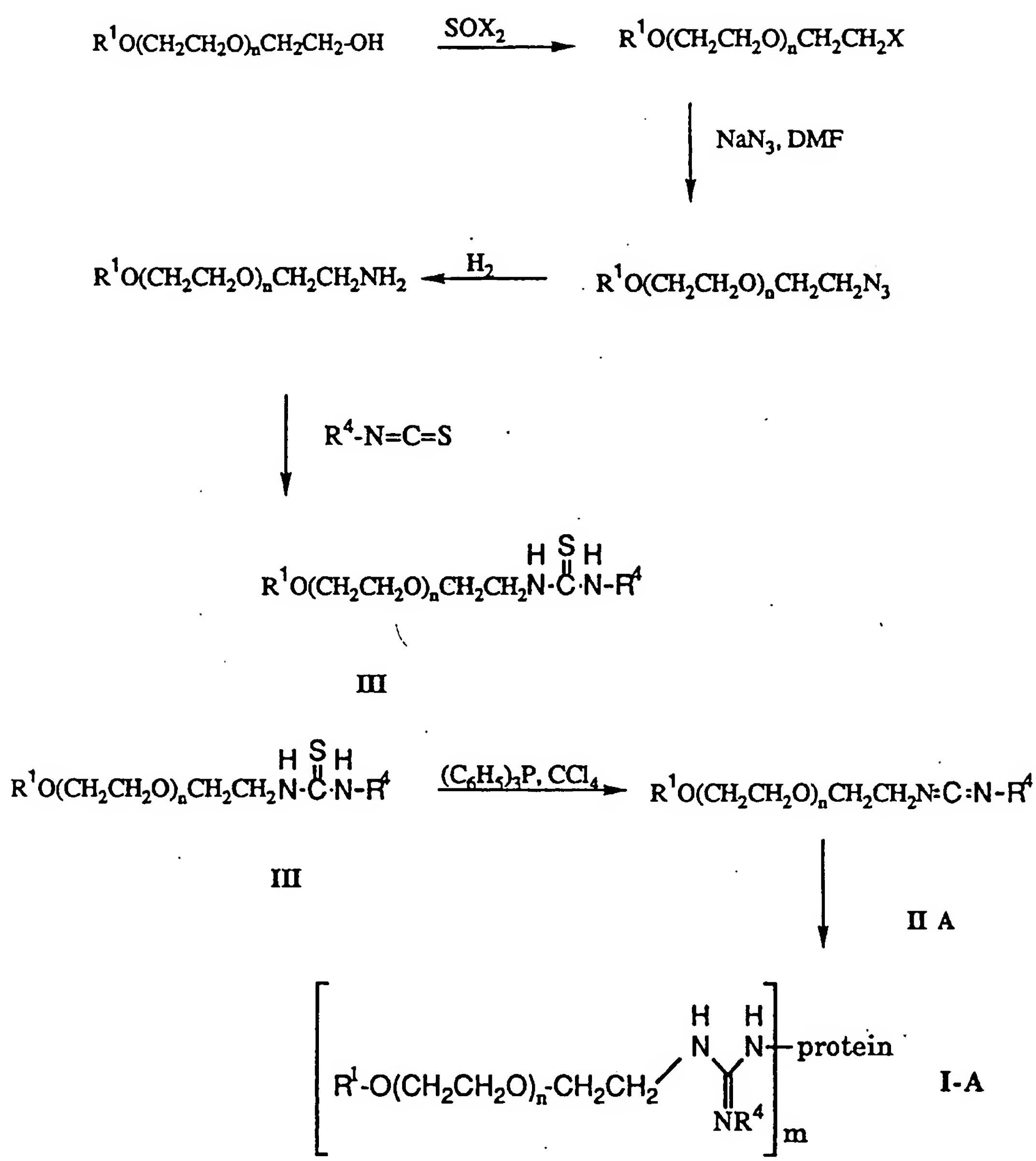
II-F 

II-G 

wherein R^1, R^5 and n are as defined above, with a free amino group of a protein or a salt thereof and isolating the protein conjugate from the reaction mixture.

In particular, the synthesis of the individual PEG derivatives to be coupled with a protein as well as their reaction with the protein can be performed as described in the following paragraphs and in the Examples.

To produce the protein conjugate wherein R^2 is -NH-, and R_3 is $=N\text{-}R^4$, (the compound of formula I-A) the following reaction scheme may be employed:



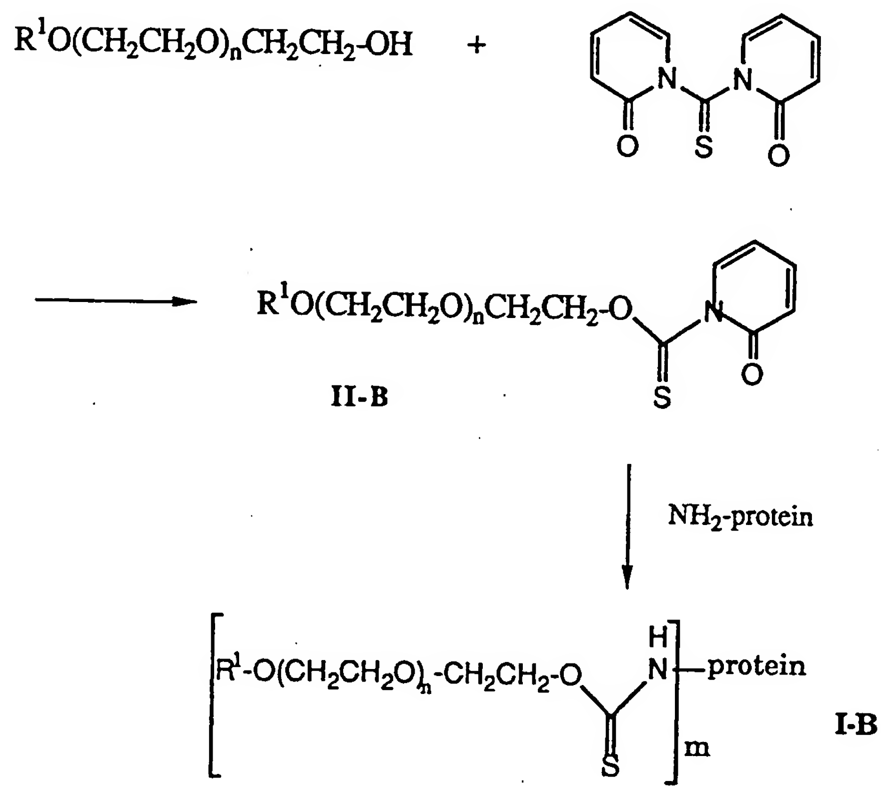
wherein R^1, R^4, n, m and the protein are as above,

In the reaction scheme, the hydroxyl group at the end of the PEG molecule is converted to a halogen group by conventional means such as by treatment with a thionyl halide. The resulting PEG halide is converted to an azide by conventional means such as by treatment with sodium azide. The PEG azide can then be converted to an amine by conventional means such as hydrogenation. The PEG-amine is then reacted with an alkyl or cycloalkyl isothiocyanate such as cyclohexylisothiocyanate to form the thiourea of formula III which is then desulfurized by conventional means to form the compound of formula II-A which contains the carbodiimide functional group. In converting the thiourea of formula III into the PEG carbodiimide of formula IIA, the preferred desulfurizing agent is triphenylphosphine.

The PEG carbodiimide of formula II-A can then be condensed with a protein under any conventional conditions for condensing carbodiimides with amines. Generally this reaction is carried out in a standard aqueous buffer solution having pH of between 7 and 9 to produce the conjugate of formula I-A. The resulting condensation reaction may produce a mixture of PEG protein conjugates of various molecular weights depending upon the number of free amino groups within the protein and the time of the reaction. The PEG protein conjugates may then be separated into their individual components by conventional

methods such as high performance liquid chromatography or gel electrophoresis.

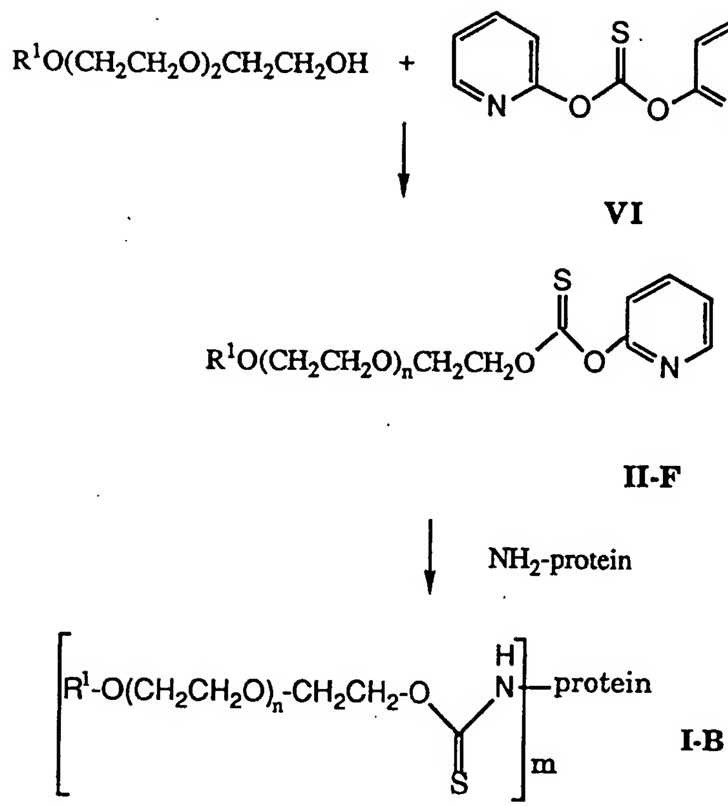
To produce the protein conjugate where R^2 is -O- and R^3 is =S (the compound of formula IB), the following reaction scheme can be employed.



wherein R^1, m, n and the protein are as above,

In this reaction a PEG is refluxed with 1,1-carbonothioylbis-2(1H)-pyridinone in a high boiling hydrocarbon solvent to produce the compound of formula II-B. The compound of formula II-B can be condensed with one or more of the free amino groups of the protein to produce the conjugate of formula I-B in the same manner as described in connection with the condensation of the compound of formula II-A with a protein to prepare the conjugate mixture of formula I-A. Separation of this mixture can be carried out according to molecular weights of the products formed as described hereinbefore.

Alternatively, the protein conjugate of the present invention where R^2 is -O- and R^3 is =S, (the compound of formula I-B) can be produced by the following reaction scheme:

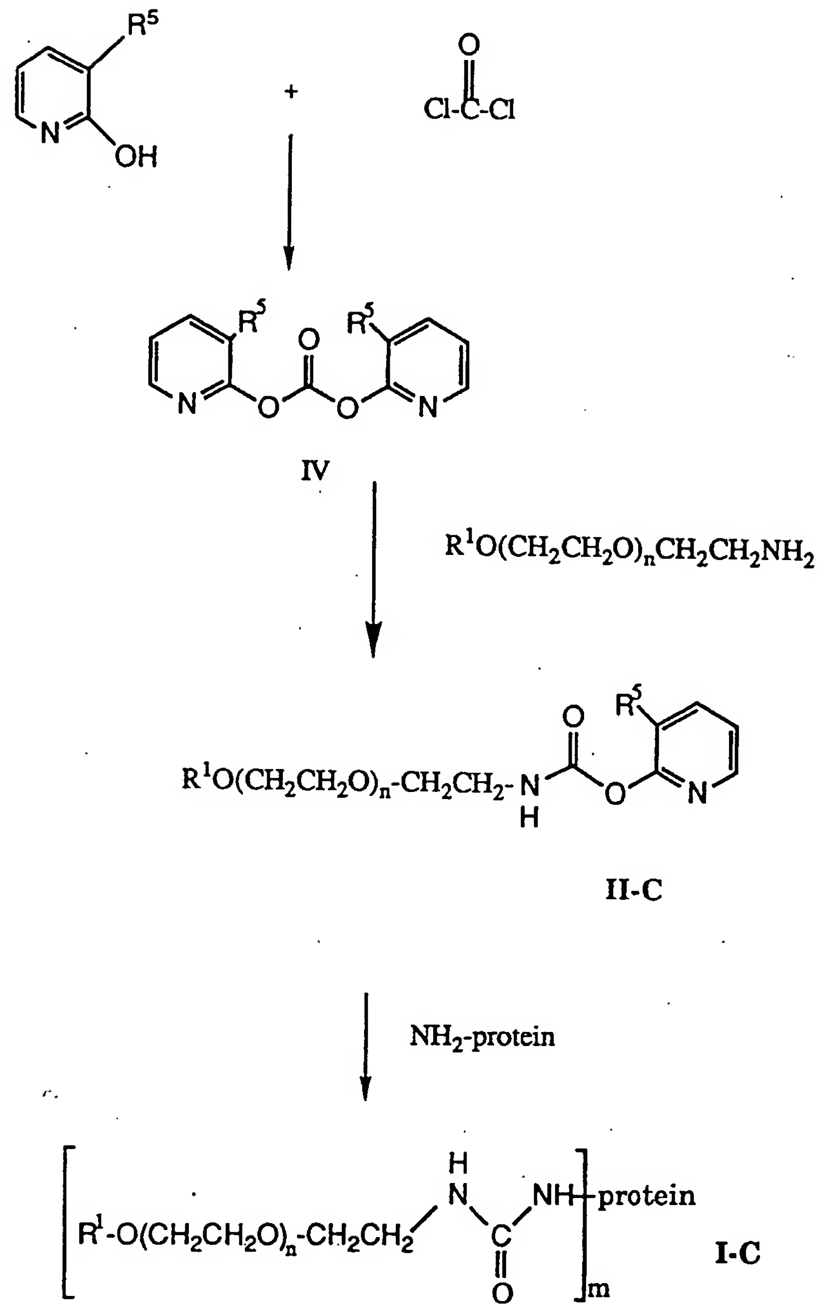


wherein R^1, m, n and the protein are as above.

In accordance with this scheme, PEG is reacted with the thiocarbonate of formula VI in an organic solvent to form the PEG thiocarbonate of formula II-F. Any conventional method of condensing a thiocarbonate with a hydroxy group can be used in carrying out this reaction.

The compound of formula II-F is converted to the conjugate of formula I-B by condensing the compound of formula II-F with at least one free amino group of the protein. This reaction is carried out in the manner described for the conversion of compound of formula II-A to the conjugate of formula I-A. The product that is produced by this reaction can be a mixture of conjugates of different molecular weights depending upon the amount of free amino groups in the protein used. These conjugates can be separated by molecular weight in accordance with the procedure hereinbefore described.

To produce the protein conjugate wherein R^2 is -NH- and R^3 is =O (the compound of formula I-C), the following reaction scheme can be used:



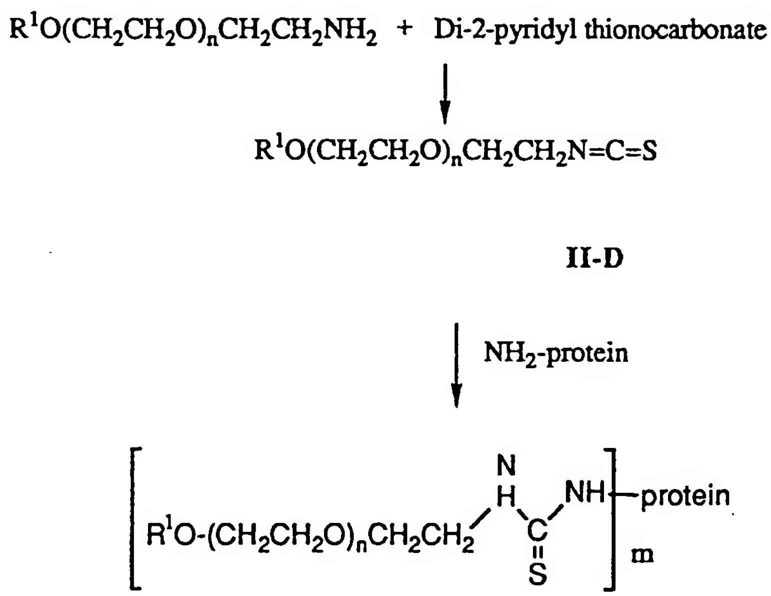
wherein R^1, R^5, m, n and the protein are as above.

The compound of formula IV is produced by condensing phosgene with 2-hydroxypyridine (substituted if R^5 = lower alkyl) using any conventional method for condensing an acid halide with an alcohol.

The condensation of a PEG amine with the compound of formula IV is effected by refluxing in a halogenated hydrocarbon solvent to produce the compound of formula II-C. The compound of formula II-C is condensed with the protein through one or more free amino groups on the protein to produce the compound of formula I-C. This reaction is carried out in the manner described for the condensation of the compound of formula II-A to produce the conjugate of formula I-A. Depending upon the number of free amino groups contained within the protein which react with the compound of formula II-C, the conjugate of formula I-C may be formed as a mixture of conjugates having different molecular weights. This conjugate

mixture can be separated in the manner described hereinbefore.

To produce a protein conjugate of the present invention wherein R^2 is -NH- and R^3 is =S (the compound of formula I-D), the following reaction scheme can be used.

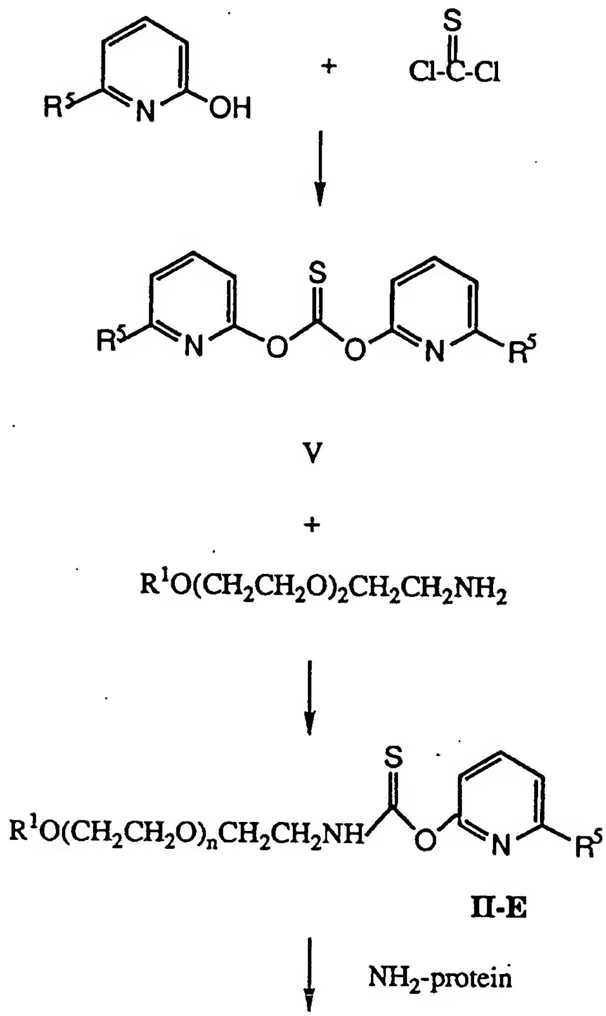


I-D

wherein R^1, m, n and the protein are as above.

In this reaction scheme, PEG amine is reacted with di-2-pyridylthionocarbonate to produce the compound of formula II-D. In this procedure any conventional method of condensing an amine with a thiocarbonate to produce an isothiocyanate can be used. The compound of formula II-D is reacted with the protein to form the conjugate of formula I-D in the manner described for the conversion of the compound of formula II-A to the compound of formula I-A. Depending upon the amount of free amino groups contained by the protein, condensation of the compound of formula II-D with the protein produces a mixture of conjugates which can be separated into their individual components in the manner hereinbefore described for the separation of the conjugate of formula I.

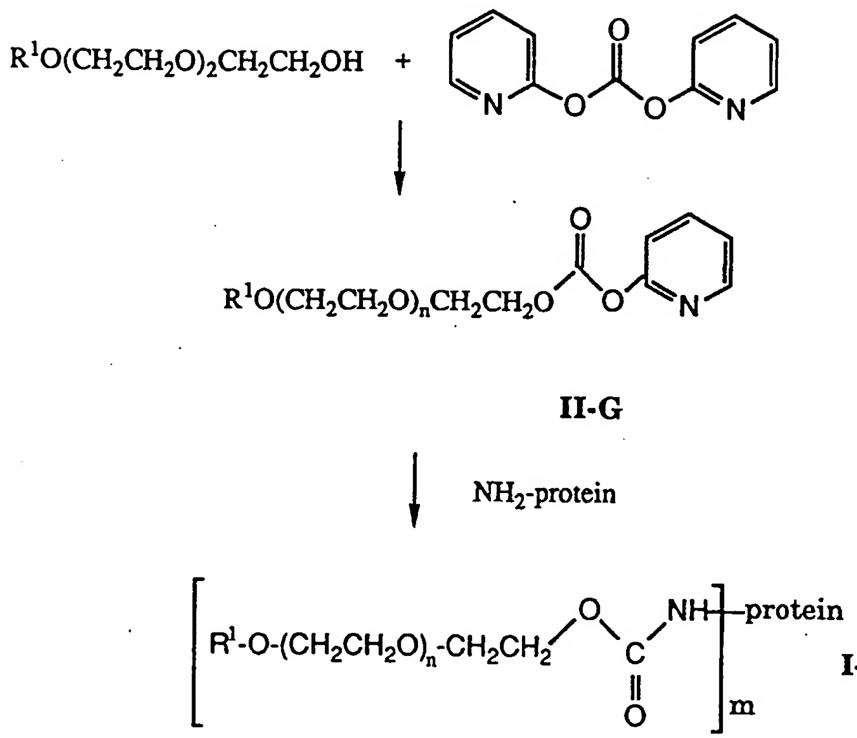
Alternatively, the compound of formula I-D can be produced using the following reaction scheme:



I-D

The compound of formula V is produced by condensing thiophosgene with 2-hydroxypyridine (substituted where R^5 is lower alkyl), using any conventional method for condensing an acid halide with an alcohol. V is then reacted with a PEG-amine in the manner described for producing the compound of II-C. The resulting compound is II-E. The compound of formula II-E is condensed with one or more free amino groups on a protein to produce the conjugate of formula I-D. This reaction is carried out in the manner described for the formation of conjugate I-A.

To produce a protein composition of the present invention wherein R^2 is -O- and R^3 is O (the compound of formula I-E), the following reaction scheme can be used.

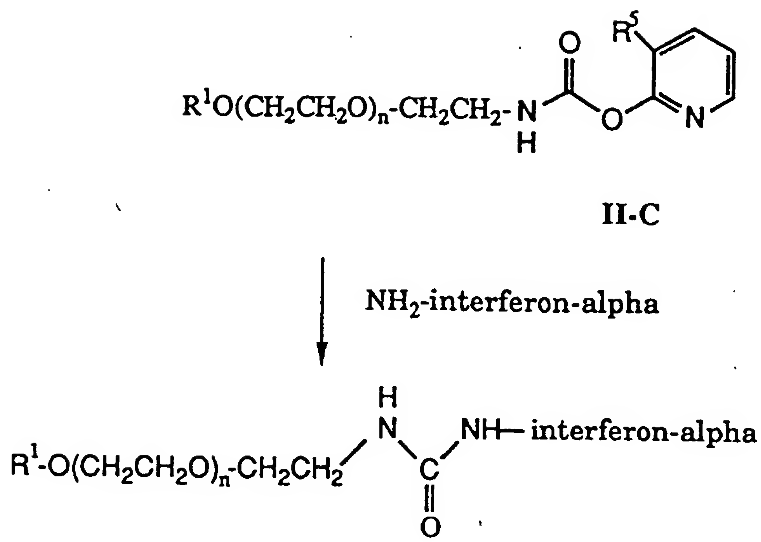


I-E

wherein R^1, m, n and the protein are as above.

In the above reaction scheme di-2-pyridyl carbonate is reacted with PEG to produce the compound of formula II-G. This reaction is carried out by conditions conventional in condensing an alcohol with a carbonate. The compound of formula II-G is converted to the compound of formula I-E by condensing the compound of formula II-G with one or more free amino groups in the protein. This reaction is carried out in the same manner as described in connection with the conversion of the compound of formula II-A to the compound of formula I-A. The reaction mixture thus produced can contain a mixture of the conjugated of formula I-E depending upon the free amino groups contained by the protein. This mixture constitutes a mixture of various conjugates of different molecular weights. The various conjugates can be separated from the mixture in accordance with procedure described hereinbefore.

In accordance with a preferred embodiment of this invention, a conjugate of an interferon-alpha with PEG can be produced by the following reaction scheme.



wherein R^1 is methyl, n is about 112. This reaction is of special interest for interferon-alpha of the subtyp interferon-alpha A (also named interferon-alpha 2).

The synthesis of the activated PEG compound II-C is described hereinbefore in connection with the synthesis of a protein conjugate wherein R^2 is -NH- and R^3 is =O. The final conjugate of interferon-alpha can be obtained in an analogous manner as described hereinbefore or in the Examples for the synthesis of the protein conjugate I-C.

Any protein with a physiological activity or a salt thereof may be used for the preparation of a conjugate with PEG provided that this protein contains at least one amino group for condensation.

Preferred proteins which can be used in the present invention are interleukin-I (IL-1), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2) and interferons, viz. IFN-alpha (leukocyte interferons), IFN-beta (fibroblast interferon) and IFN-gamma (immune interferon), their naturally occurring forms as well as analogs, homologs or truncated forms thereof.

The interleukin-1 receptor antagonist (IL-1ra) herein may be obtained from tissue cultures or by recombinant techniques. One method of obtaining Il-1ra is to reat U937 human myelomonocytic cells (ATCC CRL 1593) with the differentiating agent phorbol myristate acetate (PMA) and then stimulate them with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF; obtainable from Amgen), and isolating and purifying the IL-1ra from the culture supernatant liquid as described by Carter et al. Nature 344, 633-637 (1990).

Recombinant IL-1ra refers to IL-1ra having comparable biological activity to native IL-1ra but prepared by recombinant techniques as described by Carter et al. supra or by Eisenberg et al. Nature 343, 341-346 (1990).

Interferon includes all types of interferons, such as $\alpha,\beta,\gamma,$ or ω interferons, and any subtypes of any types. Interferons may be obtained from tissues or tissue cultures, or may be produced using recombinant techniques described in the literature, e.g. as described in European Patent Application, Publ. No. 43 980. Other methods for generating and isolating natural or recombinant interferons are also well known in the art. In accordance with this invention, it has been found that the protein-conjugates of this invention have the same utility as the protein used to form the conjugate. Therefore, these conjugates are therapeutically active in the same manner as the protein from which they are formed and can be used in the same manner as the protein itself without producing the undesired immune responses which may be connected with the administration to subjects of the proteins themselves. Therefore, the present invention also comprises the pharmaceutical compositions on the basis of the compounds of formula I or their salts and to methods for producing them.

The pharmaceutical compositions of the present invention used in the control or prevention of illnesses comprises a protein conjugate of the general formula I and a therapeutically inert, non toxic and therapeutically acceptable carrier material. The pharmaceutical compositions to be used can be formulated and dosed in a fashion consistent with good medical practice taking into consideration the disorder to be treated, the condition of the individual patient, the site of delivery of the protein conjugate, the method of

administration and other factors known to practitioners.
The following examples represent illustrative embodiments of the present invention without limiting it by them.

Example 1

Preparation of alpha-[2-[[(Cyclohexylcarbonimidoyl)-amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

A. Preparation of alpha-(2-Chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 (As used herein SRU designates the number of self-repeating units present in the PEG polymer).

From a slurry of 50 g MPEG (methoxypolyethylene glycol, molecular weight-5000) in 700 ml of toluene was distilled 200ml of the solvent. To the refluxing solution was added dropwise 0.8 ml of dry pyridine and 2.2 ml of thionyl chloride. After refluxing for four hours the reaction was allowed to stir overnight. The solvent was then removed under reduced pressure and 500ml of CH_2Cl_2 added to the residue. The resultant solution was then dried with anhydrous K_2CO_3 and passed through 50g of basic alumina (Wolem Super I). Most of the CH_2Cl_2 was then removed under reduced pressure and one liter of diethyl ether added to the resultant syrup. The ether was removed by distillation and additional diethyl ether added to cause precipitation. The mixture was stirred at room temperature for two hours and then filtered to give 45 g of alpha-(2-chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

Anal. Calcd for $C_3H_7ClO(CH_2CH_2O)_{111.7}$ C,53.97; H,9.12; Cl,0.71. Found: C, 54.21 H,8.70; Cl,0.71

B. Preparation of alpha-(2-Azidoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

A mixture of 20g of sodium azide and 50g of alpha-(2-chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 was heated at 120-125°C in 375 ml of dry DMF. After 7 hours the solvent was removed under high vacuum. The residue was dissolved in 500 ml of CH_2Cl_2 and filtered through diatomaceous earth. Most of the CH_2Cl_2 was then boiled off and diethyl ether added to cause precipitation. The mixture was stirred overnight and then filtered. The residue was then dissolved in a minimum of glyme at 50°C, the solution cooled, and the precipitated product filtered to give alpha-(2-azidoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

Anal. Calcd for $C_3H_3N_3O(CH_2CH_2O)_{111.7}$: C,53.77; H,9.09; N,0.84. Found: C,53.61; H,9.08; N,0.89.

C. Preparation of alpha-(2-Aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

To a mixture of 25g of alpha-(2-azidoethyl)omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 in 250 ml of dry glyme was added 3.5 g of 10% Pd/C. The mixture was then placed under an atmosphere of 50 p.s.i. of H_2 and shaken at 50°C for 18 hours. The mixture was then filtered, the solids washed with CH_2Cl_2 and the combined organic solutions placed under reduced pressure to remove the solvent. The residue was then dissolved in 100 ml of warm glyme, and the product allowed to precipitate from the cooled solution. The precipitate was filtered and dried by warming under reduced pressure to give 23g of alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

Anal. Calcd for $C_3H_9NO(CH_2CH_2O)_{111.7}$: C,54.43; H,9.20; N,0.28. Found: C,54.43; H,9.18; N,0.36.

Alternatively a solution of 40g of alpha-(2-azidoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 and 6.7 g (25.6 mmol) of triphenylphosphine dissolved in 200 ml of dry CH_2Cl_2 was stirred overnight under an atmosphere of argon. Water (2ml) was added and the mixture stirred an additional 12 hours. Most of the methylene chloride was removed under vacuum and 400 ml of diethyl ether added. The precipitate was filtered, washed with ether and dissolved in 300 ml of warm (50°C) glyme. The solution was allowed to stand at room temperature overnight and the resulting precipitate filtered, washed with 2x100 ml of glyme, 2x100 ml of diethyl ether and dried in a vacuum oven under a stream of N_2 to give 35g of alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

D. Preparation of alpha-[2-[[(Cyclohexylamino)thiocarbonyl]-amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7

To a solution of 4g of alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 in 60 ml of dry glyme at 40°C was added 0.1 ml of cyclohexyl isothiocyanate. The solution was allowed to stir at

40 °C for 18 hours. The mixture was then filtered and the solvent removed under high vacuum. The residue was then dissolved in 100 ml of warm glyme, the solution cooled and the resulting precipitate filtered and dried under high vacuum to give 3.5g of alpha-[2-[[(cyclohexylamino)thiocarbonyl]-amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7

Anal. Calcd for $C_{10}H_{20}N_2OS(CH_2CH_2O)_{111.7}$: C,54.25; H,9.13; N,0.54; S,0.62; Found: C,54.39; H,8.87; N,0.55; S,0.59.

E. Preparation of alpha-[2-[[(Cyclohexylcarbonimidoyl)amino]-ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

A solution of 1 g of alpha-[2-[[(Cyclohexylamino)-thiocarbonyl]amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7, 120 mg of triphenylphosphine, 90 µl of CCl_4 and 84 µl of triethylamine in 5 ml of dry CH_2Cl_2 was refluxed for 72 hr. The solvent was then removed under reduced pressure and the residue dissolved in a minimum of dry glyme. After cooling, the product precipitated and was filtered and dried under vacuum to give alpha-[2-[[(cyclohexylcarbonimidoyl)amino]ethyl]-omega-methoxypoly-(oxy-1,2-ethanediyl) SRU 111.7.

Anal. Calcd for $C_{10}H_{18}N_2O(CH_2CH_2O)_{111.7}$: C,54.61; H,9.15; N,0.55. Found: C,54.95; H,9.27; N,0.50.

Example 1a

Preparation of alpha-(2-Chloroethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225

By the procedure described in Example 1A, MPEG molecular weight 10,000 was converted to alpha-(2-chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_3H_7ClO(CH_2CH_2O)_{225}$: C,54.37; H,9.14; Cl,0.35. Found: C,54.30; H,9.15; Cl,0.41.

Example 1b

Preparation of alpha-(2-Azidoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225

By the procedure described in Example 1B, alpha-(2-chloroethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225 was converted to alpha-(2-azidoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_3H_7N_3O(CH_2CH_2O)_{225}$: C,54.34; H,9.13; N,0.42. Found: C,54.32; H,9.28; N,0.50.

Example 1c

Preparation of alpha-(2-Aminoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225

By the procedure described in Example 1C, alpha-(2-azidoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225 was converted to alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_3H_9NO(CH_2CH_2O)_{225}$: C,54.48; H,9.17; N,0.14. Found: C,54.80; H,9.21; N,0.12.

Example 1d

Preparation of alpha-(2-Chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3

To a solution of freshly distilled oxalyl chloride (0.5 ml) in 40 ml of dry CH_2Cl_2 at 0 °C was added dropwise 0.5 ml of dry DMF in 10 ml of CH_2Cl_2. The resulting solution was warmed to room temperature, stirred for 15 min and then again cooled to 0 °C. MPEG molecular weight 1325, (5.6 gr) was then added, and the resulting solution refluxed for 5 hr. The mixture was then poured into water and the mixture extracted with CH_2Cl_2. The CH_2Cl_2 solution was dried ($MgSO_4$) and the solvent removed under reduced pressure to give 1.7 g of alpha-(2-chloroethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3 as a white powder.

Anal. Calcd for $C_3H_7ClO(CH_2CH_2O)_{28.3}$: C,53.38; H,9.03; Cl,2.64. Found: C,53.48; H,9.10; Cl,2.41.

Example 1e

Preparation f alpha-(2-Azido thyl)- mega-meth xypoly (oxy-1,2-ethanediyl) SRU 28.3

By the procedure described in Example 1B, alpha-(2-chloroethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 28.3 was converted to alpha-(2-azidoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3.

Anal. Calcd for $C_3H_7N_3O(CH_2CH_2O)_{28.3}$: C,53.12; H,8.99; N,3.11. Found: C,53.21; H,9.07; N,2.98.

Example 1f

Preparation of alpha-(2-Aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3

By the procedure described in Example 1C, alpha-(2-azidoethyl)-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 28.3 was converted to alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3.

Anal. Calcd for $C_3H_9NO(CH_2CH_2O)_{28.3}$: C,54.47; H,9.17; N,0.14. Found: C,54.44; H,9.19; N,0.15.

Example 2

Preparation of Recombinant Interferon-alpha (IFN-alpha) Conjugated to PEG by means of the reagent alpha-[2-[[(Cyclohexylcarbonimidoyl)amino]-ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

alpha-[2-[[(Cyclohexylcarbonimidoyl)amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7 prepared according to Example 1 was added to 1 mg of homogenous IFN-alpha in 200 ul of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 10 moles of reagent per one mole IFN-alpha. The solutions were thoroughly mixed and the pegylation reaction allowed to proceed at room temperature for 60 minutes.

The amount of derivatization (or pegylation) of IFN-alpha was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (TABLE 1). Proteins were visualized by Coomassie Blue staining. Analysis of the products from the 60 min. reaction reveals new higher molecular weight protein species corresponding to PEG-conjugated IFN-alpha. IFN-alpha has an apparent molecular weight of 15 kD by SDS-PAGE. Unmodified IFN-alpha whose apparent molecular weight remains unchanged is therefore not conjugated with PEG. The PEG-modified IFN-alpha product has an apparent molecular weight of 28 kD.

TABLE I

Modification of IFN-alpha with the reagent described in Example 1	
Apparent Molecular Weight of IFN Protein (kD)	% of Total Protein from Reaction
15 (unmodified)	80
28	20

Example 3

Preparation of Recombinant Interleukin-2 (rIL-2) conjugated to PEG by means of the reagent alpha-[2-[[(Cyclohexylcarbonimidoyl]amino]ethyl]-omega-methyoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

Alpha-[2-[[(cyclohexylcarbonimidoyl]amino]ethyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7 prepared according to Example 1 was added to 2 mg of rIL-2 in 200 ul of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 10 moles of reagent per one mole rIL-2. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes.

The amount of derivatization (or pegylation) of the protein was estimated by SDS-PAGE (Table II). Proteins were visualized by Coomassie Blue staining. Analysis of the products from the 60 min. reaction reveals new higher molecular weight protein species corresponding to PEG-conjugated rIL-2. rIL-2 has an apparent molecular weight of 15 kD by SDS-PAGE. Unmodified rIL-2 is the protein separated from the reaction mixture whose molecular weight remains unchanged and is therefore not conjugated with PEG. The

predominant PEG-modified rIL-2 product has an apparent molecular weight of 28 kD.

TABLE II

Modification of rIL-2 with the reagent described in Example 1	
Apparent Molecular Weight of rIL-2 Protein (kD)	% of Total Protein from Reaction
15 (unmodified)	20
28	50
33	20
43	10

Pegylated rIL-2 was purified from the reaction mixture as described by Katre et al. [Proc. Nat. Acad. Sci., USA, 84:1483-1491, (1987)] using hydrophic exchange chromatography (Bio-Rad; Biogelphenyl 5-PW). A linear gradient with decreasing salt from 1.53 to 0.0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 in 30 minutes was used to separate PEG-modified and unmodified rIL-2. Aliquots of the fractions were evaluated by SDS-PAGE and pooled fractions were assayed to determine its specific activity in a CTLL cell proliferation assay by the method described by Gillis et al. [J. Immunology 120:2027-2032, (1978)]. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 0.667 for rIL-2. The specific activity of the rIL-2 isolated proteins is expressed as units/mg protein and the results are summarized in Table III. It is apparent that specific activity of rIL-2 is not significantly altered by conjugation with PEG.

TABLE III

Bioactivity of rIL-2 conjugated to PEG with the reagent described in Example 1	
Apparent Molecular Weight of rIL-2 Protein (kD)	Specific Activity (units/mg)
15 (unmodified IL2)	2.0×10^7
28	2.4×10^7

Example 4

Preparation of Recombinant Interleukin 1-alpha (rIL-1 alpha) Conjugated to PEG by means of the reagent alpha-[2-[[(Cyclohexylcarbonimidoyl]amino]ethyl]omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

The reagent described in Example 1 was added to 2.0 mg of homogenous rIL-1 alpha in 1.0 ml 0.1 M sodium borate, pH 9.0 in a molar ratio of 10 moles of reagent per one mole rIL-1 alpha. The solution was thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes.

The amount of derivatization (or pegylation) of the protein was estimated by SDS-PAGE (Table IV). The proteins were visualized by Coomassie Blue staining. Analysis of the products from the 60 minute reaction reveals new higher molecular weight protein species corresponding to PEG conjugated rIL-1 alpha protein. rIL-1 alpha has an apparent molecular weight of 17 kD by SDS-PAGE. Unmodified rIL-1 alpha is protein from the reaction mixture whose apparent molecular weight remains unchanged and is therefore not conjugated with PEG. The PEG-modified rIL-1 alpha product has an apparent molecular weight of 30 kD.

TABLE IV

Modification of rIL-1 alpha with the reagent described in Example 1	
Apparent Molecular Weight of rIL-1 alpha Protein (kD)	% of Total Protein from Reaction
17 (unmodified)	85
30	15

Example 5

Preparation of alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7

From a solution of 1 g (0.2 mmol) MPEG (methoxypolyethylene glycol) molecular weight of 5000, in 15 ml of dry toluene was distilled 5 ml of solvent. The resulting solution was cooled and 46.5 mg (0.2 mmol) of 1,1-carbonothioylbis-2(1H)-pyridinone was added. The mixture was then refluxed under an atmosphere of argon for 4 hours. The solvent was then removed under vacuum and the residue dissolved in 5 ml of dry glyme and let stand overnight. The resulting precipitate was then filtered and washed with 2x5 ml of dry glyme and 5 ml of diethyl ether. The product was then dried in a vacuum oven under a slow stream of nitrogen to give 0.96 g of alpha-[(1,2-dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7.

Anal. Calcd for $C_9H_{11}NO_3S(CH_2CH_2O)_{111.7}$: C,54.37; H,8.99; N,0.27; S,0.62; Found: C,54.03; H,8.98; N, 0.18; S,0.59.

Example 5a

Preparation of alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225

By the procedure described in Example 5, MPEG (methoxypolyethylene glycol) molecular weight 10,000 was converted to alpha-[(1,2-dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_9H_{11}NO_3S(CH_2CH_2O)_{225}$: C, 54.54; H, 9.08; N, 0.14; S, 0.32. Found: C, 54.38; H, 9.16; N, 0.15; S, 0.31.

Example 6

Preparation of Interleukin 1 receptor antagonist (IL-1ra) conjugated to PEG by means of the reagent alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxypoly-(oxy-1,2-ethanediyl) SRU 111.7.

Alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxy-poly(oxy-1,2-ethanediyl) SRU 111.7 prepared according to Example 5 was added to 10 mg of homogenous IL-1ra in 1.0 ml of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 5 moles of reagent per one mole of IL-1ra. The solution was thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes.

The amount of derivatization (or pegylation) of the protein was estimated by SDS-PAGE (Table V). Proteins were visualized by Coomassie Blue staining. Analysis of the products from the 60 minute reaction reveals new higher molecular weight protein species corresponding to PEG-conjugated IL-1ra protein. IL-1ra has an apparent molecular weight of 19 kD by SDS-PAGE. Unmodified IL-1ra is the protein from the reaction mixture whose apparent molecular weight remains unchanged and is therefore not conjugated with PEG.

The predominant PEG-modified IL-1ra proteins have apparent molecular weights of 26 and 33 kD.

TABLE V

Modification of IL-1ra with the reagent described in Example 5	
Apparent Molecular Weight of IL-1ra Protein (kD)	% of Total Protein from Reaction
19 (unmodified)	36
26	33
33	21
>33	10

Pegylated IL-1ra was purified from the reaction mixture using hydrophobic exchange chromatography (Bio-Rad; HRLC MP7 HIC). A linear gradient with decreasing salt concentrations from 0.43 to 0.0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 in 20 minutes was used to separate pegylated IL-1ra and unmodified IL-1ra. Aliquots of fractions were evaluated by SDS-PAGE and pooled fractions were assayed in an IL-1 radioreceptor competition binding assay. [Kilian et al. J. Immunol., 136, 4509-4514, (1986)]. Briefly, IL-1ra and PEG-IL-1ra were incubated at varying concentrations with EL-4 membranes for 30 min at 37°C. The $[^{125}I]$IL-1 alpha was then added and the incubation continued for 30 min. The assay was terminated by vacuum filtration and the collection of cell bound $[^{125}I]$IL-1 on filter plates. The concentration of IL-1ra or PEG-IL-1ra that inhibits binding of $[^{125}I]$IL-1 by 50% (IC_{50}) was determined graphically. The results are shown in TABLE VI. The IC_{50} of IL-1ra in this assay is 1-2.0 ng/ml. The pegylated IL-1ra mixture retained its ability to bind to the IL-1 receptor on EL-4 membranes within a factor 2 to 3 fold relative to the unmodified Il-1ra.

TABLE VI

Inhibition of $[^{125}I]$ IL-1 Binding by of IL-1ra Conjugated with the reagent described in Example 5	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50} (ng/ml)
19K (unmodified)	2.0
26K, 33K (mixture)	5.0

The pharmacodynamics of IL-1ra protein was evaluated in vivo by the ability of the IL-1ra to inhibit rIL-1alpha induction of interleukin-6. Serum from mice treated with rIL-1alpha contain high levels of IL-6, [McIntosh et al., Immunol. 143: 162-167, (1989)]. The administration of unmodified IL-1ra together with IL-1alpha (0 hr time point) inhibits the induction of IL-6. This test system was used to compare the pharmacodynamic properties of unmodified and PEG IL-1ra. Groups of three female C57B1/6 mice were injected subcutaneously with 200 ug of unmodified IL-1ra or PEG-IL-1ra 48 hr, 24 hr, or 6 hr before or simultaneously (0 hr) with 0.2 ug rIL-1 alpha. Three hours later, serum samples were collected. IL-6 levels (units) were determined using a modification of an IL-6 assay that has been previously described [Van Snick et al., Proc. Natl. Acad. Sci. USA 83:9679-9683, (1986)]. In the IL-6 assay, B9 hybridoma cells were treated with two fold serial dilutions of the test sera in 96-well microtiter plates. Following a 3 day incubation at 37°C in a humidified atmosphere comprised of 5% CO_2 and 95% air, the wells were pulsed with 0.5 uCi of tritiated thymidine and incubated for an additional 18 hr. The cells were then harvested onto glass fiber filters and the level of tritiated thymidine incorporation was determined by scintillation counting. IL-6 activity is expressed as U/ml. IL-6 units are defined as the inverse of the serum dilution which produces half-maximal tritiated thymidine incorporation compared to a reference standard.

The pharmacodynamic data are summarized in Table D1. Mice treated only with IL-1 exhibited 28852 U/ml IL-6. Both unmodified and modified IL-1ra inhibited IL-6 induction at 0 hr. However, the pegylated IL-1ra demonstrated a prolonged IL-6-inhibitory effect as compared to unmodified IL-1ra at 8 and 24 hours after administration.

TABLE D1

Pharmacodynamic Profile of IL-1ra Conjugated with the reagent described in Example 5		
Time (hr.) Prior to IL-1 Administration	IL-6 (Units/ml)	
	19 kD	26 kD
0	772	705
6	8361	1587
24	22525	9844
48	18485	21119
72	13220	21470

Example 6A

Preparation of Interleukin 1 receptor antagonist (IL-1ra) conjugated to PEG by means of the reagent alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly-(oxy-1,2-ethanediyl) SRU 225.

IL-1ra was conjugated and purified according to the procedure illustrated in Example 6 using the reagent described in Example 5a.

The predominant PEG-modified IL-1ra proteins have apparent molecular weights of 33 kD and 48 kD. The 33kD and 48 kD protein accounted for 46 and 27% of the total protein in the reaction mixture, respectively.

The ability of these proteins to inhibit IL-1 binding, as described in Example 6, is summarized in Table VII. The 33kD PEG modified protein retains the ability to inhibit IL-1 binding within 6-fold relative to IL1-ra and more extensive modification of the protein with PEG as observed with this 48kD protein results in a substantial loss in its binding to the IL-1 receptor.

TABLE VII

Inhibition of [^{125} I] IL-1 Binding by IL-1ra Proteins Conjugated with the reagent described in Example 5a	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50}(ng/ml)
19 (unmodified)	1.6
33	9.0
48	50.0

To determine the pharmacokinetic profile of PEG-IL-1ra species, C57BF/6 mice were administered 100 ug of modified or pegylated IL-1ra species subcutaneously. Serum samples were collected after 1, 2, 3, 4 and 6 hours from mice that received unmodified IL-1ra and after 2, 4, 8, 10, and 24 hours from mice that received PEG-IL-1ra. The serum levels were determined in the EL4 membrane binding assay described in Example 6. The data are summarized in Table D2. The PEG-IL-1ra was detectable in serum samples at higher concentrations and for prolonged time compared to IL-1ra demonstrating a prolonged serum time course.

TABLE D2. Pharmacokinetic Profile of IL-1ra pegylated as in Example 6a

Time (hr.) After Administration	Serum Concentration of IL-1ra 19 kD	33 kD
1	400	
2	130	2500
.	40	
4	15	800
6	16	
8		300
10		250
24		15

Example 7

Preparation of rIL-1 alpha conjugated to PEG by means of the reagent alpha-[(1,2-dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxy-poly(oxy-1,2-ethanediyl) SRU 111.7.

Recombinant IL-1 alpha was pegylated with the reagent described in Example 5 by the method as described in Example 4. Three predominant molecular weight species from the reaction mixture were identified by SDS-PAGE with apparent molecular weights corresponding to 17 (unmodified), 26, and 33 kD. The latter two pegylated proteins accounted for 25 and 55% of the total protein, respectively.

Pegylated rIL-1 alpha was purified from the reaction mixture after 60 minutes using hydrophobic exchange chromatography (Bio-Rad; HRLC MP7 HIC). A linear gradient with decreasing salt concentration from 0.43 to 0.0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 in 20 minutes was used to separate pegylated rIL-1 alpha and unmodified rIL-1 alpha. Aliquots of fractions were evaluated by SDS-PAGE and pooled fractions were assayed for specfic activity in a D10 cell proliferation assays by the method described by Kaye et al. [J. Exp. Med. 158:836-854 (1983)]. Protein concentrations were determined spectrophotometrically at 280 nM using an extinction coefficient of 1.0 for rIL-1 alpha. The specific activity of the rIL-1 alpha is approximately1.0 x 10^8 units/mg. The specific activity results are summarized in Table VIII. The 26kD pegylated IL-1 alpha conjugate retains bioactivity within 2-3 fold of relative to IL-1 alpha. Further modification resulting in a 33kD protein results in substantial loss of bioactivity.

TABLE VIII

Bioactivity of rIL-1alpha Conjugated with the reagent described in Example 5	
Apparent Molecular Weight of rIL-1 alpha Protein (kD)	Specific Activity (units/mg)
17 (unmodified)	4.6 x 10^7
26	1.9 x 10^7
33	4.5 x 10^6

Example 8

Pr parati n of IFN-alpha conjugated to PEG by m ans of alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)-thiocarb nyl]- mega-methoxypoly(oxo-1,2-ethanediyl) SRU 111.7

alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7 prepared according to Example 5, was added to 1 mg of purified IFN-alpha in 100 ul of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 8 moles of the PEG reagent per one mole IFN-alpha. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes.

The predominant molecular weight species from the reaction mixture were identified by SDS-PAGE with apparent molecular weights of 15 (unmodified), and 28 kD. The 28 kD pegylated protein accounted for 40% of the total protein. The pegylated IFN-alpha was purified from the 60 minute reaction mixture and characterized using hydrophobic exchange chromatography (Bio-Rad; Biogel-phenly-S-PW). A linear gradient with decreasing salt concentrations from 0.42M to 0.0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 in 20 minutes was used to separate pegylated IFN-alpha and unmodified IFN-alpha. Aliquots of the fractions were evaluated by SDS-PAGE and pooled fractions were assayed for anti-viral activity (specific activity) in an MDBK assay by the method described in by Familletti, et al. [Methods Enzym. 78,387-394 (1987)].

Protein concentrations were determined spectrophotometrically at 280 nM using an extinction coefficient of 1.0 for a 1 mg/ml IFN-alpha buffered solution. The specific activity of the isolated proteins is expressed as units per mg protein and the results are summarized in Table IX.

The results show that the specific activity of the 28kD pegylated IFN-alpha was not significantly altered relative to IFN-alpha.

TABLE IX

Bioactivity of IFN-alpha Conjugated with the reagent of Example 5	
Apparent Molecular Weight of IFN-alpha Protein (kD)	Specific Activity (units/mg)
15 (unmodified)	1.1×10^8
28	1.4×10^8

Example 8A

Preparation of IFN-alpha conjugated to PEG by means of the reagent alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxypoly-(oxy-1,2-ethanediyl) SRU

IFN-alpha was pegylated as in example 8 with the reagent described in Example 5a. Three predominant molecular weight species from the reaction mixture at 60 minutes were identified by SDS-PAGE with apparent molecular weights corresponding to 15 (unmodified), 35 and 43 kD. The latter two pegylated proteins accounted for 35 and 33 per cent of the total proteins in the reaction mixture, respectively.

The specific activities determined by procedures described in Example 8 of the pegylated species of IFN-alpha are summarized in Table X. The results show that the 35kD pegylated IFN-alpha product retained biological activity within 2-3 fold of IFN-alpha. The 43kD conjugate lost substantial activity.

TABLE X

Bioactivity of IFN-alpha Conjugated with the reagent of Example 5a	
Apparent Molecular Weight of IFN-alpha Protein (kD)	Specific Activity (units/mg)
15 (unmodified)	3.3×10^8
35	1.2×10^8
43	1.5×10^7

Example 8B

Pr paration of rIL-2 conjugat d to PEG by means f the reagent alpha-[(1,2-Dihydro-2- xo-1-pyridinyl)thiocarb nyl]omega-methoxypoly(oxy-1,2-ethanediyl) SRU 111.7

rIL-2 was pegylated with the reagent described in Example 5 and purified using the procedure as described in Example 3.

The predominant molecular weight species from the reaction mixture after 60 minutes were identified by SDS-PAGE with an apparent molecular weights of 15 (unmodified) and 25 kD. The 25 kD pegylated protein accounted for 60% of the total protein in the reaction.

The specific activity of the rIL-2 isolated proteins were measured as described in Example 3 and is expressed as units/mg protein and the results are summarized in Table XI.

As can be see in Table XI, the biological activity of IL-2 was not altered after conjugation with PEG.

TABLE XI

Bioactivity of rIL-2 Conjugated to PEG with the reagent described in Example 5	
Apparent Molecular Weight of rIL-2 Protein (kD)	Specific Activity (units/mg)
15 (unmodified)	2.0×10^7
25	2.0×10^7

Example 8C

Preparation of rIL-2 conjugated to PEG by means of the reagent alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxypoly(oxy-1,2-ethanediyl) SRU

rIL-2 was pegylated using the procedure described in Example 3 with the reagent described in Example 5a.

The predominant molecular weight species from the reaction mixture after 60 minutes were identified by SDS-PAGE with apparent molecular weights of 15 kD (unmodified), 33 kD, and 43 kD. The 33 and 43 kD pegylated proteins accounted for 60 and 20 per cent of the total protein in the reaction, respectively.

Example 9

Preparation of IFN-alpha conjugated to PEG by means of the reagent alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]omega-methoxy-poly(oxy-1,2-ethanediyl) SRU 111.7.

An alternative method for conjugating IFN-alpha to PEG was done as follows:

IFN-alpha (5 mg in 1 ml) was dialyzed against a buffer containing 5 mM sodium acetate, pH 5.0, 120 mM NaCl. To the dialyzed protein solution, solid potassium thiocyanate was added to obtain a final concentration of 0.5 M salt, and the pH adjusted by the addition of one-tenth volume of 1 M tricine-sodium hydroxide, pH 11.9 to give a final pH 10.0 solution. alpha-[(1,2-Dihydro-2-oxo-1-pyridinyl)thiocarbonyl]-omega-methoxypoly(oxy-1,2-ethanediyl) was added to the sample at a molar ratio of 3 moles of reagent to 1 mole of protein. The modification reaction was allowed to proceed at room temperature for 30 minutes, and stopped by the addition of 1 M glycine, pH 6.3 to a final concentration of 20 mM. PEG-modified protein was precipitated from solution by addition of a buffer containing 3.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 to a final concentration of 1.1 M ammonium sulfate and the precipitate collected by centrifugation (10,000 x g for 12 min.). After rinsing the pellet with a buffer containing 1.1 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0, the pellet was redissolved in a buffer containing 25 mM ammonium acetate, pH 5.0. The PEG-modified protein was purified and characterized as described in Example 2. A single pegylated IFN species was obtained with an apparent molecular weight of 28 kD. Antiviral activity (specific activity) of the modified protein was determined by the procedure described in Example 8. The specific activity of the starting IFN-alpha was 2.6×10^8 U/mg and the specific activity of the IFN-alpha conjugated to PEG was 1.0×10^8 U/mg demonstrating that the PEG conjugated IFN-alpha retained biological activity within 3-fold relative to IFN-alpha.

Example 10

Preparation of IFN-alpha conjugat d to PEG by means f the reag nt alpha-[(1,2-Dihydr -2-oxo-1-pyridinyl)-thiocarbonyl]-omega-meth xypoly(oxy-1,2-ethanediyl) SRU 225.

IFN-alpha was conjugated to PEG according to the procedure described in Example 9. The proteins were purified and characterized as described in Examples 2 and 9. The starting IFN-alpha had a specific activity of 2.6 x 10^8 U/mg using the IFN-alpha conjugated to PEG which has an apparent molecular weight of 31 kD and had a specific activity of 1.0 x 10^8 U/mg as described in Example 8. The bioactivity of the conjugated IFN-alpha was within 3-fold of IFN-alpha.

Example 11

Preparation of alpha-Methyl-omega-[2-[[(2-pyridinyloxy) carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 111.7.

From a solution of 1 g (0.2 mmol) of alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 (as prepared in Example 1C) in 40 ml of dry CH_2Cl_2 was distilled 15 ml of solvent. To the resulting solution at 0°C was then added 65 mg (0.3 mmol) of di-2-pyridyl carbonate and the mixture stirred for an additional 4 hours. The solvent was then removed under reduced pressure and the residue triturated with diethyl ether. The precipitate was then filtered and washed with 50 ml of ether followed by 50 ml of hexane. The product was then dried in a vacuum oven under a slow stream of nitrogen to give 1 g of alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino] ethoxy]poly(oxy-1,2-ethanediyl) SRU 111.7. as a white powder.

Anal. Calcd for $C_9H_{12}N_2O_3(CH_2CH_2O)_{111.7}$: C,54.56; H,9.04; N,0.55; Found: C,54.26; H,9.00; N,0.53.

Example 11a

Preparation of alpha-Methyl-omega-[2-[[(2-pyridinyloxy) carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225

By the procedure described in Example 11, alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225, (as prepared in Example 1c) was converted to alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_9H_{12}N_2O_3(CH_2CH_2O)_{225}$: C, 54.54, H, 9.10; N, 0.28. Found: C, 54.49; H, 9.27; N, 0.31.

Example 11b

Preparation of alpha-Methyl-omega-[2-[[(2-pyridinyloxy) carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 28.3

By the procedure described in Example 11, alpha-(2-amino-ethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 28.3 (as prepared in Example 1f) was converted to alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]-amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 28.3.

Anal: Calcd for $C_9H_{12}N_2O_3(CH_2CH_2O)28.3$: C, 54.61; H, 8.75; N, 1.94. Found: C, 54.67; H, 8.96; N, 1.63.

Example 12

Preparation of IL-1ra conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7

alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 111.7, was added to 25 mg of purified IL-1ra in 2.5 ml of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 1 mole of reagent per one mole of IL-1ra. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes. PEG modified ILra was then purified according to the procedure set out in Example 6.

The predominant pegylated products from the 60 minute reaction had apparent molecular weights of 28 kD and 38 kD and accounted for approximately 42 and 29% of the total protein from the reaction mixture, respectively.

The ability of the purified IL-1ra proteins from the reaction mixture to inhibit IL-1 binding was

determined as described in Example 6 and summarized in Table XII. The binding properties of the 28kD product was not significantly altered and the bindability of the 38kD protein retained activity within 5-fold of IL-1ra.

TABLE XII

Inhibition of [^{125}I]-IL-1 Binding by IL-1ra Protein Pegylated with the reagent described in Example 11	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50} (ng/ml)
19 (unmodified)	2.0
28	3.0
38	10.0

The pharmacodynamic profile of PEG-IL-1ra was determined as described in Example 6. The data are summarized in Table D3. IL-1 alone induced 27283 u/ml of IL-6. Unmodified IL-1ra inhibited less than 50% of the IL-1 response within 6 hours of administration. In contrast, PEG IL-1ra although less active at early time points, was much more active at 24 and 48 hours after injection. Thus, the PEG-IL-1ra exhibited a prolonged pharmacodynamic profile.

TABLE D3

Pharmacodynamic Profile of IL-1ra Conjugated with the reagent described in Example 11		
Time (hr.) Prior to IL-1 Administration	IL-6 Units/ml	
	19 kD	26kD
0	4789	23806
6	15324	10833
24	24841	5727
48	16348	9364
72	12067	12054

Example 12A

Preparation of IL-1ra conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 225

alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225, (previously described in Example 11a) was added to 25 mg of purified IL-1ra in 2.5 ml of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 4 moles of reagent per one mole of IL-1ra. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes. PEG modified IL-1ra was then purified according to the procedure set out in Example 6.

The predominant pegylated products from the 60 minute reaction had apparent molecular weights of 33 kD and 48 kD and accounted for approximately 76 and 15% of the total protein from the reaction mixture, respectively. The ability of the purified IL-1ra proteins from the reaction mixture to inhibit IL-1 binding are summarized in Table XIII. The 33kD PEG modified protein retained its ability to inhibit IL-1 binding within 8-fold relative to IL-1ra. The 48kD product lost substantial binding capacity.

TABLE XIII

Inhibition of [^{125}I]IL-1 Binding by IL-1ra Proteins Pegylated with the reagent described in Example 11a	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50} (ng/ml)
19 (unmodified)	0.8
33	6.0
48	18.0

The pharmacokinetic profile of PEG-IL-1ra was determined as described in Example 6A. The data are summarized in Table D4. The PEG-IL-1ra was detectable in serum samples at higher concentrations and for a prolonged time compared to the unmodified IL-1ra.

TABLE D4

Pharmacokinetic Profile of IL-1ra Conjugated with the reagent described in Example 11a		
Time (hr.) After Administration	Serum Level of IL-1ra (ng/ml)	
	19 kD	33 kD
1	220	
2	33	700
3	13	
4	5.3	500
6	1.5	
8		150
10		83
24		5

Example 13

Preparation of rIL-2 conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7

rIL-2 was pegylated with alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7, according to the procedure set forth in Examples 3 and 8b. The specific activity of the IL-2 protein was determined as described in Example 8. The specific activity of the 15 kD unmodified rIL-2 was 2 x 10^7 units/mg and of the 29 kD pegylated IL-2 was 2.4 x 10^7 units/mg IL-2 indicating no substantial loss of biological activity as a result of pegylation.

Example 14

Preparation of PEG-modified rIL-1 alpha conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7

rIL-1 alpha was pegylated with the reagent described in Example 11, alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 111.7, according to the procedure set forth in Examples 4 and 7. Two pegylated rIL-1 alpha proteins with apparent molecular weights of 28 kD and 38 kD were purified and accounted for 50 and 25 per cent of the total proteins from the reaction mixture at 60 minutes respectively.

Example 15

Preparation f IFN-alpha conjugated to PEG by means of th reag nt alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly](xy-1,2-ethanediyl), SRU 111.7

IFN-alpha was pegylated with the reagent described in Example 11, alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 111.7, according to the procedure set forth in Example 8. Forty percent of the protein was derivatized after 60 minutes and the product had an apparent molecular weight of 26 kD.

Example 16

Preparation of IFN-alpha conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)-carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7 Alternative method of peyglating IFN-alpha.

Using the procedure illustrated in Example 9, IFN-alpha was conjugated to PEG by alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl), SRU 111.7. The specific activity of IFN-alpha was determined as described in Example 8. The specific activity of the starting IFN-alpha was 1.7×10^8 U/mg and the specific activity of the IFN-alpha conjugated to PEG by alpha-methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) was 0.8×10^8 U/mg which is within 2-3 fold of the IFN-alpha.

Example 17

Preparation of IFN-alpha conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225.

Using the procedure illustrated in Example 9, IFN-alpha was pegylated by means of the reagent described in Example 11a. The specific activity as determined by the method described in Example 8 of the IFN-alpha conjugated to PEG was 0.4×10^8 U/mg demonstrating no significant loss in bioactivity .

Example 18

Preparation of alpha-[(2-Pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

From a solution of 1 g MPEG molecular weight 5000 dissolved in 30 ml of dry CH_2Cl_2 was distilled 10 ml of solvent. The solution was cooled to room temperature and 132 mg (0.6 mM) of di-2-pyridyl carbonate and 4 mg of DMAP were added. The resulting solution was then stirred for 14 hours and the solvent removed under vacuum. The residue was triturated with diethyl ether and the resulting precipitate filtered. The product was then dissolved in 7 ml of dry glyme, warmed to cause dissolution, and the resulting solution allowed to cool and stand at room temperature for several hours. The resulting precipitate was then filtered and washed with 2x5 ml of dry glyme. The solid was then dried in a vacuum oven and under a stream of nitrogen to give 0.7 g of alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7.

Anal. Calcd for $C_9H_{11}NO_4(CH_2CH_2O)_{111.7}$: C, 54.57; H, 9.02; N, 0.28. Found: C, 54.51; H, 9.19; N, 0.28.

Example 18a

Preparation of alpha-[(2-Pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225

By the procedure described in Example 18, MPEG (methoxypolyethylene glycol) molecular weight 10,000 was converted to alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225.

Anal. Calcd for $C_9H_{11}NO_4(CH_2CH_2O)_{225}$: C, 54.54; H, 9.08; N, 0.14. Found: C, 54.54; H, 9.12; N, 0.11.

Exampl 19

Preparation of IL-1ra conjugated to PEG by means f th reagent alpha-[(2-Pyridinyloxy)carbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

IL-1ra was pegylated with alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7 by the procedure previously described in Examples 6 and 12. The predominant pegylated products had apparent molecular weights of 26 kD and 33 kD and accounted for approximately 31 and 57

per cent of the total protein from the 60 minute reaction mixture, respectively. The ability of the purified IL-1ra proteins from the reaction mixture to inhibit IL-1 binding was determined as described in Example 6 and summarized in Table XIV. The 26kD pegylated IL-1ra conjugate retained its binding capacity within 4-fold of IL-1ra. The 33kD conjugate lost significant binding activity as indicated by a 15-fold decrease in competitive binding activity.

TABLE XIV

Inhibition of [^{125}I]IL-1 Binding by IL-1ra Proteins Pegylated with the reagent described in Example 18	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50}(ng/ml)
19 (unmodified)	2.0
26	8.0
33	30.0

Example 19A

Preparation of IL-1ra conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)carbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225

IL-ra was pegylated with alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225, according to the procedure set forth in Example 19. The predominant pegylated products from the 60 minute reaction method mixture had apparent molecular weights of 33 kD and 48 kD and accounted for approximately 47 and 25 per cent of the total protein from the reaction mixture, respectively.

The ability of the purified IL-1ra proteins from the reaction mixture to inhibit IL-1 binding was determined as described in Examples 6 and 12 and summarized in Table XV. The 33kD protein retained activity within 6-fold of IL-1ra. The higher molecular weight conjugate lost significant activity.

TABLE XV

Inhibition of [^{125}I]IL-1 Binding by IL-1ra Proteins Pegylated with the reagent described in Example 18a	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50}(ng/ml)
19 (unmodified)	1.5
33	9.0
48	40.0

The pharmacokinetic profile of PEG-IL-1ra was determined as described in Example 6A. The data are summarized in Table D5. The PEG-IL-1ra was detectable in the serum samples for a prolonged time compared to the unmodified IL-1ra demonstrating a prolonged serum half-life. The pharmacodynamic profile of PEG-IL-1ra was determined as described in Example 6, except that 0.05 ug of rIL-1 alpha was administered. The data are summarized in Table D6. The response to IL-1 alone was 9203 units/ml IL-6. A prolonged inhibitory effect, compared to unmodified IL-1ra, can be seen at up to 72 hours following administration of the PEG-IL-1ra demonstrating improved pharmacodynamic properties. Collectively, these data illustrate that even if a pegylated protein has diminished activity in vitro, it could have improved pharmaco-dynamic properties in vivo.

TABLE D5

Pharmacokinetic Profile of IL-1ra Conjugated with the reagent described in Example 18a		
Time (hr.) After Administration	Serum IL-1ra (mg/ml)	
	19 kD	33 kD
1	580	
2	75	100
3	30	
4	30	60
6	8	
8		12
10		17
24		10

TABLE D6

Pharmacodynamic Profile of IL-1ra Conjugated with the reagent described in Example 18		
Time (hr.) Prior to IL-1 Administration	IL-6 (Units/ml)	
	19 kD	26 kD
0	521	454
6	2334	416
24	13486	2552
48	16577	4667
72	12800	5148

Example 20

Preparation of IFN-alpha conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)-carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

The reagent alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7, was added to 1 mg of purified IFN-alpha in 200 ul of buffer (0.1 sodium borate, pH 9.0) in a molar ratio of 10 moles of reagent per mole IFN-alpha. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes. Purified PEG-modified IFN-alpha was then obtained according to the procedure set forth in Example 8. Thirty-six percent of the protein was derivatized and the product had an apparent molecular weight of 28 kD.

The specific activity of the purified IFN proteins from the reaction mixture were determined as described in Example 8 and the values are summarized in Table XVI. The modified IFN had a 5-6 fold decrease in biological activity in vitro.

TABLE XVI

Bioactivity of IFN-alpha Conjugated with the reagent described in Example 18	
Apparent Molecular Weight of IFN-alpha Protein (kD)	Specific Activity units/mg
15 (unmodified)	1.88×10^8
28	8.0×10^7

Example 20A

Preparation of rIL-2 conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)carbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

The reagent alpha-[(2-pyridinyloxy)carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7, previously described in Example 18, was added to 1 mg of rIL-2 in 200 ul of buffer (0.1 sodium borate, pH 9.0) in a molar ratio of 5 moles of the reagent per one mole of rIL-2. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes.

The predominant molecular weight species from the 60 minute reaction mixture were identified by SDS-PAGE with apparent molecular weights of 15 kD (unmodified) and 25 kD. The 25 kD pegylated protein accounted for 60% of the total protein.

Example 21

Preparation of IFN alpha conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)-carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7.

IFN-alpha was pegylated with the reagent described in Example 18 according to the procedure described in Example 9.

The specific activity as determined by methods described in Example 8, of the starting unmodified IFN-alpha was 1.0×10^8 U/mg and the specific activity of the IFN-alpha conjugated to PEG was 0.4×10^8 U/mg demonstrating no significant loss in bioactivity.

Example 22

Preparation of IFN alpha conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)-carbonyl]omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

PEG was conjugated to IFN-alpha using the reagent described in Example 18a with the procedures of Example 9. The specific activity as determined by methods described in Example 8 of the IFN-alpha conjugated to PEG was 0.3×10^8 U/mg.

Example 23

Preparation of alpha-[2-(Isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7

To 2 g of alpha-(2-aminoethyl)-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7 in 100 ml CH_2Cl_2 was added 94.2 mg of di-2-pyridylthionocarbonate. The solution was allowed to stir 18 hours and then extracted with a small amount of cold water. Most of the CH_2Cl_2 was removed under reduced pressure and ether added to cause precipitation. The product was filtered and dried under high vacuum to give alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7.

Anal. Calcd for $C_4H_7NOS\ (CH_2CH_2O)_{111.7}$: C, 53.88; H, 9.07; N, 0.28; S, 0.64. Found: C, 54.45; H, 8.93; N, 0.28; S, 0.53.

Example 24

Preparation IFN-alpha conjugated to PEG by means of the reagent alpha-[2-(Isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

The reagent alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7, previously described in Example 23 was added to 1 mg of purified IFN-alpha in 200 ul of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 10 moles of reagent per one mole IFN-alpha. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes. Thirty percent of the product was derivatized and had an apparent molecular weight of 26 kD.

Exampl 25

Preparati n of rIL-2 conjugated to PEG by means of th reagent alpha-[2-(Isothiocyanato)ethyl]-omega-methoxyp ly (oxy-1,2- thanediyl), SRU 111.7

The reagent alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 111.7, previously described in Example 23, was added to 1.0 mg of recombinant IL-2 (rIL-2) in 100 ul of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 10 moles of reagent PEG per mole rIL-2. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperatures for 60 minutes. Purified PEG-modified rIL-2 was then obtained according to the procedure as set forth in Example 3. The derivatization results are summarized in Table XVII.

TABLE XVII

Modification of rIL-2 with the reagent described in Example 23	
Apparent Molecular Weight of rIL-2 Protein (kD)	% of Total Protein from Reaction
15 (unmodified)	70
26	20
30	10

Example 26

Preparation IL-1ra conjugated to PEG by means of the reagent alpha-[2-(Isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

IL-1ra was pegylated with alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7, according to the procedure described in Example 6. The predominant pegylated products had molecular weights of 26, 31, 38 and 48 kD and accounted for approximately 17, 44, 15 and 10 per cent of the total protein, respectively.

The ability of the purified 26 kD IL-1ra protein from the 60 minute reaction mixture to inhibit IL-1 binding was determined by methods described in Example 6 and summarized in Table XVIII. The pegylated protein retained its binding capacity within 2-3 fold of IL-1ra.

TABLE XVIII

Inhibition of [^{125}I]IL-1 Binding by IL-1ra Protein Pegylated with the reagent described in Example 23	
Apparent Molecular Weight of IL-1ra Protein (kD)	IC_{50}(ng/ml)
19	2.0
26	5.0

Example 27

Preparation of rIL-1 alpha conjugated to PEG by means of the reagent alpha-[2-(Isothiocyanato)-ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7

Recombinant IL-1 alpha was pegylated with alpha-[2-(isothiocyanato)ethyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 111.7, as described in Example 4. Two predominant molecular weight pegylated species from the 60 minute reaction mixture were identified by SDS-PAGE with apparent molecular weights of 26 and 38 kD. The latter two pegylated proteins accounted for 46 and 48 per cent of the total protein, respectively. The pegylated rIL-1 alpha was purified from the reaction mixture and characterized as described in Example 4.

The bioactivity of the pooled purified fractions were evaluated in the D10 cell proliferation assay as described in Example 7 and results summarized in Table XIX. The samples noted as mixtures in the table

contained more than one protein species that was not further purified. The 26kD pegylated protein had a specific activity essentially indistinguishable from IL-1.

TABLE XIX

Bioactivity of rIL-1 alpha conjugated to PEG with the reagent described in Example 23	
Apparent Molecular Weight of rIL-1 alpha Protein (kD)	Specific Activity units/mg
17	1.1×10^8
26	1.7×10^8
26, 38 (mixture)	2.0×10^8
>38 (mixture)	6.0×10^6

Example 28

Preparation of alpha-[(2-Pyridinyloxy)thiocarbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

From a solution of 1 g (0.1 mmol) MPEG (methoxypolyethylene glycol molecular weight 10,000) in 30 ml of CH_2Cl_2 was distilled 10 ml of solvent. The resulting solution was cooled and 69.7 mg (0.3 mmol) of di-2-pyridyl thionocarbonate and 2 mg of DMAP added. The mixture was then stirred under an atmosphere of argon for 18 hours. The solvent was removed under vacuum and the residue redissolved in a minimum of CH_2Cl_2. Ether was then added and the resulting precipitate filtered and washed with ether. The product was then dissolved in 5 ml of warm glyme and the resulting solution allowed to stand overnight. The resulting precipitate was then filtered and washed with 2x5 ml of glyme and 5 ml of diethyl ether. The product was then dried in a vacuum oven under a slow stream of nitrogen to give 0.9 g of alpha-[(2-pyridinyloxy)thiocarbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_9H_{11}NO_3S(CH_3CH_2O)_{225.3}$: C, 54.46; H, 9.04. Found: C, 54.67; H, 9.30.

Example 29

Preparation of IL-1ra conjugated to PEG by means of the reagent alpha-[(2-Pyridinyloxy)-thiocarbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225

alpha-[(2-Pyridinyloxy)thiocarbonyl]-omega-methoxypoly (oxy-1,2-ethanediyl), SRU 225, prepared as described in Example 28, was added to 2.0 mg of IL-1ra in 1.0 ml of buffer (0.1 M sodium borate, pH 9.0) in a molar ratio of 2 moles of the reagent per one mole of IL-1ra. The solutions were thoroughly mixed and the pegylation reaction was allowed to proceed at room temperature for 60 minutes. PEG modified IL-1ra was then purified according to the procedure set out in Example 6.

The predominant pegylated product had an apparent molecular weight of 33 kD and accounted for approximately 17% of the total protein from the 60 minute reaction mixture. The ability of the purified IL-1ra proteins from the reaction mixture to inhibit IL-1 binding was determined as described in Example 6 and summarized in Table XX. The binding capacity of the 33kD protein was within 3-4 fold of the IL-1ra.

TABLE XX

Inhibition of [^{125}I]IL-1 Binding by IL-1ra Proteins pegylated with the reagent described in Example 28	
Molecular Weight of IL-1ra Protein (kD)	IC_{50}(ng/ml)
19 (unmodified)	1.4
33	5.0

Example 30

Carbonothioic acid o,o-di-(6-methyl-2-pyridinyl)ester

To a solution of 10 g (0.09 mol) of 6-methyl-2-hydroxypyridine in 250 ml of dry CH_2Cl_2 was added 12.7 ml of triethylamine. The solution was cooled to 0°C and under an atmosphere of argon was added dropwise 4.1 ml (0.046 mol) of a solution of thiophosgene in CCl_4 (85%). The mixture was then allowed to stir at room temperature for 5 hr., filtered and the CH_2Cl_2 solution washed twice with 100 ml of a saturated $NaHCO_3$ solution. The organic layer was dried and the solvent removed under reduced pressure. Hexane (100 ml) was then added to the residue and the resulting mixture was allowed to digest overnight. The resulting precipitate was filtered, washed with hexane and dried in a vacuum oven under a slow stream of nitrogen to give 5.7 g of carbonothioic acid o,o-di-(6-methyl-2-pyridinyl) ester m.p. 155-156°C.

Anal. Calcd for $C_{13}H_{12}N_2O_2S$: C, 59.98; H, 4.65; N, 10.76; S, 12.32. Found: C, 59.65; H, 4.59; N, 10.75; S, 12.06.

Example 31

alpha-Methyl-omega-[2-[[(6-methyl-2-pyridinyloxy) thiocarbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225

A solution of 1 g of alpha-methoxy-omega-(2-aminoethyl) poly(oxy-1,2-ethanediyl) SRU 225 (as prepared in Example 1c) and 52.7 mg of carbonothioic acid o,o-di(6-methyl-2-pyridinyl)ester (Example 30) dissolved in 15 ml of dry CH_2Cl_2 was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue triturated with diethyl ether. The precipitate was filtered and washed with ether. The product was then dissolved in 5 ml of warm glyme and filtered through a 0.75 micron Millipore filter. The solution was then allowed to stand at room temperature for 48 hr. and the resulting precipitate filtered. The product was then dried in a vacuum oven under an atmosphere of N_2 for 18 hr. to give 0.9 g of alpha-methyl-omega-[2-[[(6-methyl-2-pyridinyloxy)thiocarbonyl]amino] ethoxy]poly-(oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_{10}H_{14}N_2O_2S(CH_2CH_2O)_{225}$: C, 54.50; H, 9.09; N, 0.27; S, 0.32. Found: C, 54.38; H, 9.20; N, 0.21; S, 0.37.

Example 32

Preparation rIL-1ra Conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(6-methyl-2-pyridinyloxy)thiocarbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225

The reagent prepared as previously described in Example 31 was added to 5.0 mg of purified rIL-1ra in 1.0 ml 0.1 M sodium borate, pH 9.0 in a molar ratio of 2.0 moles reagent per mole rIL-1ra. The solution was thoroughly mixed and the reaction mixture was allowed to proceed at room temperature for 60 minutes.

The rIL1ra products were evaluated using the procedure described in Example 6. Table XXI shows the percent of modification of primary molecular weight species from the reaction mixture.

TABLE XXI

Modification of rIL-1ra with the reagent described in Example 31	
Apparent Molecular Weight of rIL-1ra Protein (kD)	% of Total Protein from Reaction
19 (unmodified)	30.0
35	65.0
48	5.0

rIL-1ra products from the reaction mixture were purified using the method described in Example 6.

Purified fractions from the reaction mixture were assayed in an rIL-1 radioreceptor competition binding assay as described previously in Example 6. The results are shown in Table XXII. These results show that the 35 kD protein was 6-fold less active than unmodified IL-1ra for inhibiting Il-1 binding while the 48 kD protein was 20-fold less active.

TABLE XXII

Inhibition of [125I] IL-1 binding by rIL-1ra conjugated with the reagent described in Example 31	
Apparent Molecular Weight of rIL-1ra Protein (kD)	IC_{50} (ng/ml)
19 (unmodified)	1.5
35	9.0
48	30.0

Example 33

alpha-[2-[[(2-Pyridinyloxy)carbonyl]amino]ethyl]-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]-ethoxy-[poly(oxy-1,2-ethanediyl) SRU 180

A. Preparation of alpha-(2-Chloroethyl)-omega-(2-chloroethoxy)poly(oxy-1,2-ethanediyl) SRU 180

From a slurry of 80 g MPEG (polyethylene glycol-molecular weight 8000) in 750 ml of toluene was distilled 200 ml of the solvent. To the refluxing solution was added dropwise 1.7 ml of dry pyridine and 4.7 ml of thionyl chloride. After refluxing for twelve hours the reaction was allowed to stir overnight. Methylene chloride (50 ml) was then added and the resulting solution filtered through 60 g of basic alumina (Wolem Super 1). The column was then eluted with 500 ml of CH_2Cl_2, the organic layers combined, and the solvent removed under reduced pressure. The residue was then dissolved in 300 ml of CH_2Cl_2 and ether slowly added while the solvents are removed on a steam bath. This procedure is continued until a cloudy suspension develops. The resulting mixture is then stirred at room temperature for several hours and the product then filtered to give 73 g of alpha-(2-chloroethyl)-omega-(2-chloro-ethoxy)-poly(oxy-1,2-ethanediyl) SRU 180.

Anal. Calcd for $C_2H_4Cl_2(CH_2CH_2O)180$: C, 54.16; H, 9.09; Cl, 0.88. Found: C, 53.40; H, 8.81; Cl, 0.93.

B. Preparation of alpha-(2-Azidoethyl)-omega-(2-azidoethoxy)poly(oxy-1,2-ethanediyl) SRU 180

A mixture of 72 g of alpha-(2-chloroethyl)-omega-(2-chloroethoxy)poly(oxy-1,2-ethanediyl) SRU 180, 25 g sodium azide and 700 ml of dry DMF was stirred and heated at 125°C for 12 hours. The solvent was then removed under high vacuum and the residue dissolved in one liter of CH_2Cl_2 and filtered through Celite. The CH_2Cl_2 was then removed on a steam bath while diethyl ether was added to cause precipitation. The mixture was stirred overnight and then filtered. The precipitate was then dissolved in a minimum of glyme at 50°C, slowly cooled and filtered. The product then dried in a vacuum oven under a stream of N_2 to give 69 g of alpha-(2-azidoethyl)-omega-(2-azidoethoxy)-poly(oxy-1,2-ethanediyl) SRU 180.

Anal. Calcd for $C_2H_4N_6(CH_2CH_2O)_{180}$: C, 54.07; H, 9.08; N, 1.044. Found: C, 53.76; H, 9.28; N, 0.96.

C. Preparation of alpha-(2-Aminoethyl)-omega-(2-aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 180

A solution of 69 g of alpha-(2-azidoethyl)-omega-(2-azidoethoxy)poly(oxy-1,2-ethanediyl) SRU 180 and 6.7 g (25.6 mmol) of triphenylphosphine dissolved in 200 ml of dry CH_2Cl_2 was stirred overnight under an atmosphere of argon. Water (2 ml) was added and the mixture stirred an additional 12 hours. Most of methylene chloride was removed under vacuum and 400 ml of diethyl ether added. The precipitate was filtered, washed with ether and dissolved in 300 ml of warm (50°C) glyme. The solution was allowed to stand at room temperature overnight and the resulting precipitate filtered, washed with 2x100 ml of glyme, 2x100 ml of diethyl ether and dried in a vacuum oven under a stream of N_2 to give 66 g of alpha-(2-aminoethyl)-omega-(2-aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 180

Anal. Calcd for $C_2H_8N_2(CH_2CH_2O)_{180}$: C, 54.42; H, 9.18; N, 0.35. Found: C, 53.85; H, 9.20; N, 0.43.

D. alpha-[2-[[(2-Pyridinyloxy)carbonyl]amino]ethyl]omega[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly-(oxy-1,2-ethanediyl) SRU 180

From a solution of 1 g of alpha-(2-aminoethyl)-omega-(2 aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 180 dissolved in 40 ml of dry CH_2Cl_2 was distilled 15 ml of solvent. The solution was cooled to 0°C and 85 mg

(6.39 mmol) of di-2-pyridyl carbonate added. The mixture was then stirred at 0°C for 4 hr and the solvent removed under vacuum. The residue was triturated with diethyl ether and the product filtered and washed with ether (2x75 ml). The product was then dried under vacuum and dissolved in 8 ml of dry glyme (50°C). The solution was then allowed to cool and stand at room temperature for 12 hr. The precipitated product was filtered, washed with 2 x 5 ml of glyme and dried in a vacuum oven under a stream of N_2 to give 1 g of alpha-[2-[[(2-pyridinyloxy)carbonyl] amino]ethyl]-omega-[2-[[(2-pyridinyloxy)carbonyl] amino]ethoxy] poly-(oxy-1,2-ethanediyl) SRU 180.

Anal. Calcd for $C_{14}H_{14}N_4O_4(CH_2CH_2O)_{180}$: C, 54.57; H, 8.99; N, 0.68. Found: C, 54.32; H, 8.79; N, 0.77.

Example 34

alpha-[2-[[(2-Pyridinyloxy)carbonyl]amino]ethyl]-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]-ethoxy]poly(oxy-1,2-ethanediyl) SRU 452

A. Preparation of alpha-(2-Chloroethyl)-omega-(2-chloroethoxy)-poly(oxy-1,2-ethanediyl) SRU 452

By the procedure described in Example 33A, polyethylene glycol molecular weight 20,000 was converted to alpha-(2-chloroethyl)-omega-(2-chloroethoxy)-poly(oxy-1,2-ethanediyl) SRU 452.

Anal. Calcd for $C_2H_4Cl_2(CH_2CH_2O)452$: C, 54.38; H, 9.13; Cl, 0.35. Found: C, 54.36; H, 9.23; Cl, 0.40.

B. Preparation of alpha-(2-Azidoethyl)-omega-(2-azidoethoxy) - poly(oxy-1,2-ethanediyl) SRU 452

By the procedure described in Example 33B, alpha-(2-chloroethyl)-omega-(2-chloroethoxy)-poly(oxy-1,2-ethanediyl) SRU 452 was converted to alpha-(2-azidoethyl)-omega-(2-azidoethoxy)-poly-(oxy-1,2-ethanediyl) SRU 452.

Anal. Calcd for $C_2H_4N_6(CH_2CH_2O)_{452}$: C, 54.35; H, 9.12; N, 0.42. Found: C, 54.38; H, 9.30; N, 0.47.

C. Preparation of alpha-(2-Aminoethyl)-omega-(2-aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 452

By the procedure described in Example 33C, alpha-(2-azidoethyl)-omega-(2-azidoethoxy)-poly(oxy-1,2-ethanediyl) SRU 452 was converted to alpha-(2-aminoethyl)-omega-(2-aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 452.

Anal. Calcd for $C_2H_8N_2(CH_2CH_2O)_{452}$: C, 54.49; H, 9.17; N, 0.14. Found: C, 54.44; H, 9.19; N, 0.15.

D. alpha-[2-[[(2-Pyridinyloxy)carbonyl]amino]ethyl]-omega-[2-[[(2-pyridinyloxy)car-bonyl]amino]ethoxy]poly-(oxy-1,2-ethanediyl) SRU 452.

By the procedure described in Example 33D, alpha-(2-aminoethyl)-omega-(2-aminoethoxy)poly(oxy-1,2-ethanediyl) SRU 452 was converted to alpha-[2-[[(2-pyridinyloxy)carbonyl]amino]ethyl]-omega-[2-[[(2-pyridinyloxy)car-bonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 452.

Anal. Calcd for $C_{14}H_{14}N_4O_4(CH_2CH_2O)_{452}$: C, 54.56; H, 9.08; N, 0.28. Found: C, 54.33; H, 9.13; N, 0.33.

Example 35

Preparation rIL-1ra Conjugated to PEG by means of the reagent alpha-[2-[[(2-pyridinyloxy)-carbonyl]amino]ethyl]-omega-[2-[[(2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 452

The reagent prepared as previously described in Example 34 was added to 5.0 mg of purified rIL-1ra in 1.0 ml 0.1 M sodium borate, pH 9.0 in a molar ratio of 1.0 moles of reagent per 4.0 moles rIL-1ra. The solution was thoroughly mixed at room temperature for 60 minutes.

The rIL-1ra products were evaluated using the method previously described in Example 6. Table XXIII shows the percent of modification of primary molecular weight species from the reaction mixture.

Example 33

Apparent Molecular Weight of rIL-1ra Protein (kD)	% of Total Protein from Reaction
19 (unmodified)	50.0
55	35.0
75	15.0

Example 36

Bis-(3-Methyl-2-pyridyl)carbonate

A solution of 4.6 g (42 mmol) of 3-methyl-2-hydroxypyridine and 6 ml of triethylamine dissolved in 20 ml of CH_2Cl_2 was added dropwise at 0°C to a solution of 50 ml of CH_2Cl_2 and 11 ml of phosgene in toluene (1.93 molar). The mixture was stirred at 0°C for 2 hr. and then at room temperature of 2 hr. The reaction mixture was then washed with a saturated $NaHCO_3$ solution followed by a saturated NaCl solution and then dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue crystallized from EtOAc/hexane to give 3 g of bis-(3-methyl-2-pyridyl) carbonate m.p. 110-112°C.

Anal. Calcd for $C_{13}H_{12}N_2O_3$: C, 63.93; H, 4.95; N, 11.47. Found: C, 63.78; H, 4.86; N, 11.23.

Example 37

alpha-Methyl-omega-[2-[[(3-methyl-2-pyridinyloxy) carbonyl]amino]ethoxy]poly(oxy-1,2-ethane-diyl) SRU 225

From a solution of 1 g of alpha-methoxy-omega-(2-aminoethyl)poly(oxy-1,2-ethanediyl) SRU 225 dissolved in 25 ml of CH_2Cl_2 was distilled 10 ml of solvent. To the solution was then added 49.2 mg (0.2 mmol) of bis-(3-methyl-2-pyridyl)carbonate and the resulting mixture stirred overnight under an atmosphere of argon. The solvent was then removed under reduced pressure and 80 ml of diethyl ether added to the residue. The solid was filtered, washed with ether and then dissolved in 10 ml CH_2Cl_2. Ether was then slowly added while boiling off the solvent until the solution becomes turbid. The mixture was then allowed to stand for 18 hr. at room temperature and the resulting precipitate filtered. The solid was then dissolved in 8 ml of warm glyme and allowed to sit at room temperature for an additional 18 hr. The product was then filtered and dried in a vacuum oven under an atmosphere of nitrogen to give 0.6 g of alpha-methyl-omega-[2-[[(3-methyl-2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225.

Anal. Calcd for $C_{10}H_{14}N_2O_3(CH_2CH_2O)_{225}$: C, 54.59; H, 9.09; N, 0.28. Found: C; 55.64; H, 9.14; N, 0.22.

Example 38

Preparation rIL-1ra Conjugated to PEG by means of the reagent alpha-Methyl-omega-[2-[[(3-methyl-2-pyridinyloxy)carbonyl]amino]ethoxy]poly(oxy-1,2-ethanediyl) SRU 225

The reagent prepared as previously described in Example 37 was added to 5.0 mg of purified rIL-1ra in 1.0 ml 0.1 M sodium borate, pH 9.0 in a molar ratio of 2.0 moles reagent per mole rIL-1ra. The solution was thoroughly mixed and the reaction was allowed to proceed at room temperature for 60 minutes.

The rIL-1ra products were evaluated using the procedure described in Example 6. Table XXIV shows the percent of modification of primary molecular weight species from the reaction mixture.

TABLE XXIV

Modification of rIL-1ra with the reagent described in Example 37	
Apparent Molecular Weight of rIL-1ra Protein (kD)	% of Total Protein from Reaction
19 (unmodified)	45.0
35	43.0
45	12.0

rIL-1ra products from the reaction mixture were purified as described in Example 6. Purified fractions from the reaction mixture were assayed in an rIL-1 radioreceptor competition binding assay as described in Example 6. The results are shown in Table XXV. The 35 kD protein was 4-fold less active than unmodified IL-1ra for inhibiting IL-1 binding. The 45 kD protein was 30-fold less active.

TABLE XXV

Inhibition of [^{125}] IL-1 binding by rIL-1ra conjugated with the reagent described in Example 35	
Apparent Molecular Weight of rIL-1ra Protein (kD)	IC_{50} (ng/ml)
19 (unmodified)	1.0
35	4.0
45	30.0

Claims

1. A physiologically active conjugate of a protein of the formula

$$\left[R^1\text{-O-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}R^2\text{-}\underset{\underset{\displaystyle R^3}{\|}}{C}\text{-NH} \right]_m\text{-protein} \qquad \mathrm{I}$$

wherein R^1 is lower alkyl; R^2 is -O- or -NH-; R^3 is $=N\text{-}R_4$, $=S$ or $=o$; R^4 is lower alkyl or cycloalkyl; m and n are selected from integers ≥ 1 in a way that the conjugate has at least a portion of the biological activity of the non conjugated protein;

with the proviso that when R^2 is -O-, R^3 is other than $=N\text{-}R^4$;

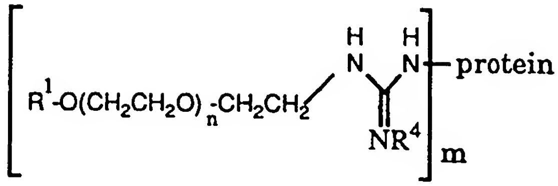
that when R^2 is -O- and R^3 is $=O$ the protein is interferon-alpha, interleukin-1 or interleukin-1ra and

that when R^2 is -O- and R^3 is $=S$ the protein is not antigen E from ragweed pollen or bovine plasma albumin.

2. A protein conjugate of claim 1 wherein m and n are selected in a way that the molecular weight of the pegylation residue(s) per protein molecule is from 300 to 30,000 daltons.

3. A protein conjugate of claim 1 or claim 2 wherein m is 1 or 2.

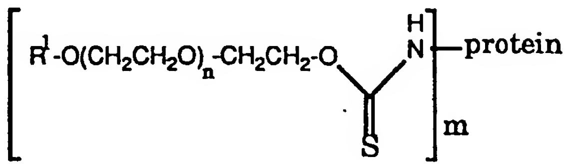
4. A protein conjugate of any one of claims 1-3 of the formula



I-A

wherein R^1, R^4, m, n and the protein are as in claim 1.

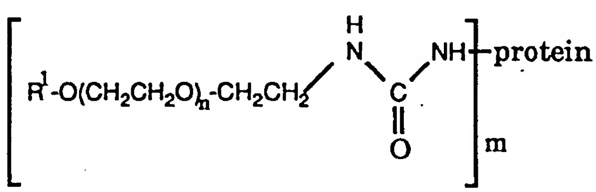
5. A protein conjugate of any one of claims 1-3 of the formula



I-B

wherein R^1, m, n and the protein are as in claim 1.

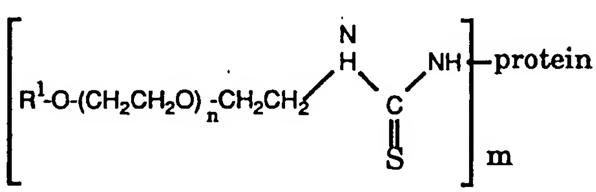
6. A protein conjugate of any one of claims 1-3 of the formula



I-C

wherein R^1, m, n and the protein are as in claim 1.

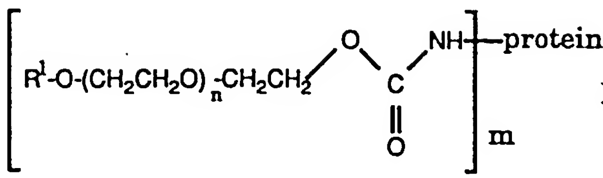
7. A protein conjugate of any one of claims 1-3 of the formula



I-D

wherein R^1, m, n and the protein are as in claim 1.

8. A protein conjugate of any one of claims 1-3 of the formula



I-E

wherein R^1, m, n and the protein are as in claim 1.

9. A protein conjugate according to any one of claims 1-8 wherein R^1 is CH_3.

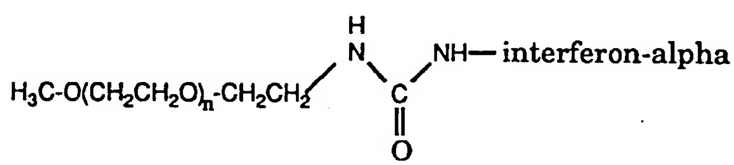
10. A protein conjugate according to any one of claims 1-9 wherein the protein is interleukin-1.

11. A protein conjugate according to any one of claims 1-9 wherein the protein is interleukin-1ra.

12. A protein conjugate according to any one of claims 1-9 wherein the protein is interferon-alpha.

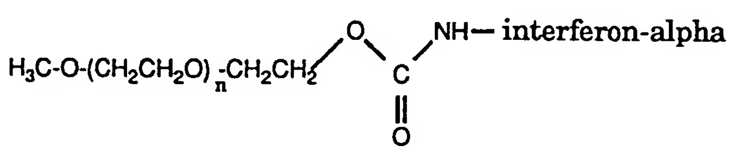
13. A protein conjugate according to any one of claims 1-9 wherein the protein is interleukin-2.

14. The protein conjugate of claim 1 of the formula



wherein n is about 112.

15. The protein conjugate of claim 1 of the formula



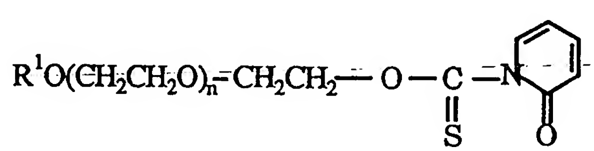
wherein n is about 112.

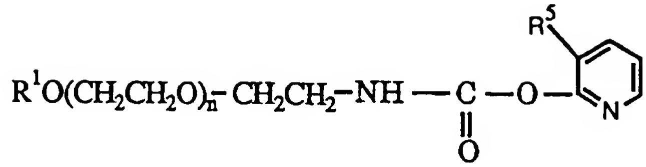
16. The protein conjugate according to claim 12 or claim 13 wherein the interferon-alpha is interferon-alpha A.

17. A compound selected from the group consisting of

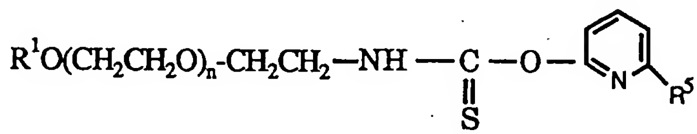
II-A $R^1O(CH_2CH_2O)_n-CH_2CH_2-N=C=N-R^4$

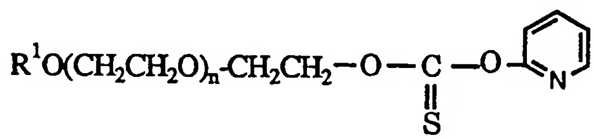
II-B

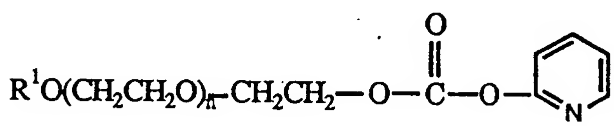


II-C 

II-D $R^1O(CH_2CH_2O)_n\text{-}CH_2\text{-}CH_2\text{—}N=C=S$

II-E 

II-F 

II-G 

wherein R^1 is lower alkyl, R^4 is lower alkyl or cycloalkyl, R^5 is lower alkyl or H and n is any integer of from 1 to 1000.

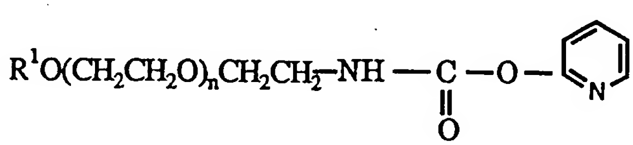
18. A compound of claim 17 wherein R^4 is cyclohexyl.

19. A compound of claim 17 wherein R^5 is H.

20. A compound of claim 17 wherein R^5 is CH_3.

21. A compound according to any one of claims 17-20 wherein R^1 is CH_3.

22. A compound with the formula



wherein R^1 is CH_3 and n is about 112.

23. Protein conjugates in accordance with any one of claims 1-16 for use as therapeutically active compounds in the treatment or prophylaxis of illnesses.

24. Process for the preparation of a protein conjugate claimed in any one of claims 1-16, which process comprises reacting a compound claimed in claim 17 with a free amino group of a protein or a salt thereof and isolating the protein conjugate from the reaction mixture.

25. Pharmaceutical compositions comprising a protein conjugate as claimed in any one of claims 1-16 and a therapeutically inert carrier.

26. Pharmaceutical compositions for the treatment or prophylaxis of immunomodulatory disorders such as neoplastic diseases or infectious diseases comprising a protein conjugate as claimed in any one of claims 1-16 and a therapeutically inert carrier.

27. The use of the protein conjugates according to any one of claims 1-16 in the treatment or prophylaxis of illnesses of others than human beings.

28. The use of the protein conjugates according to any one of claims 1-16 for the manufacture of medicaments for use in the treatment or prophylaxis of illnesses.

Claims for the following Contracting States : GR, ES

1. A process for the preparation of a physiologically active conjugate of a protein of the formula

$$\left[R^1\text{-O-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}R^2\text{-}\underset{\underset{R^3}{\|}}{C}\text{-NH} \right]_m\text{-protein} \qquad \mathbf{I}$$

wherein R^1 is lower alkyl; R^2 is -O- or -NH-; R^3 is $=N\text{-}R_4$, $=S$ or $=O$; R^4 is lower alkyl or cycloalkyl; m and n are selected from integers ≥ 1 in a way that the conjugate has at least a portion of the biological activity of the non conjugated protein;

with the proviso that when R^2 is -O-, R^3 is other than $=N\text{-}R^4$;

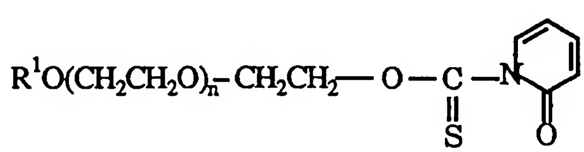
that when R^2 is -O- and R^3 is $=O$ the protein is interferon-alpha, interleukin-1 or interleukin-1ra and

that when R^2 is -O- and R^3 is $=S$ the protein is not antigen E from ragweed pollen or bovine plasma albumin;

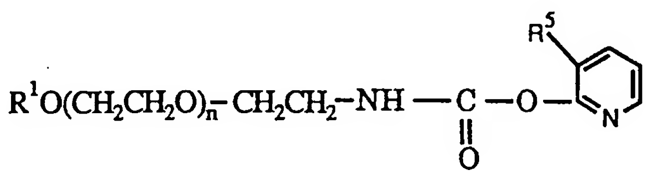
wich process comprises reacting one of the activated compounds of the general formulae

$$\mathbf{II\text{-}A} \quad R^1O(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}N{=}C{=}N\text{-}R^4$$

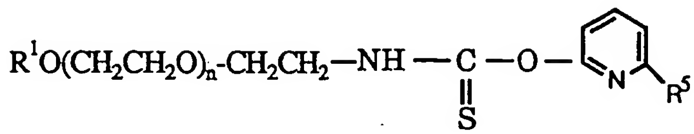
II-B

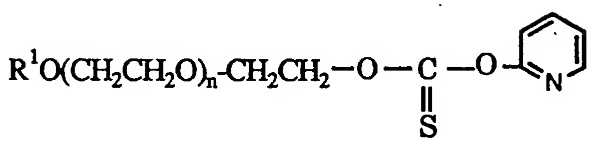


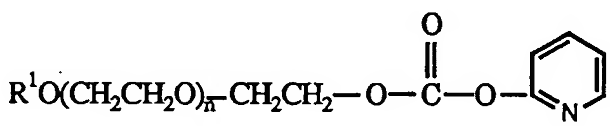
II-C



$$\mathbf{II\text{-}D} \quad R^1O(CH_2CH_2O)_n\text{-}CH_2\text{-}CH_2\text{-}N{=}C{=}S$$

II-E 

II-F 

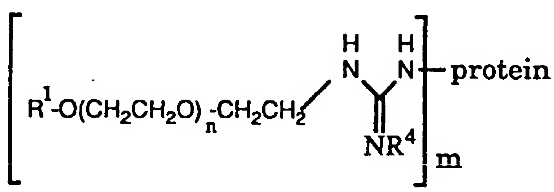
II-G 

wherein R^1, R^4 and R^5 are as defined above and n is any integer of from 1 to 1000, with a free amino group of a protein or a salt thereof and isolating the protein conjugate from the reaction mixture.

2. A process as claimed in claim 1 wherein m and n are selected in a way that the molecular weight of the pegylation residue(s) per protein molecule is from 300 to 30,000 daltons.

3. A process according to claim 1 or claim 2 wherein m is 1 or 2.

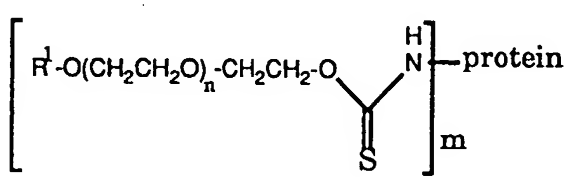
4. A process of any one of claims 1-3 for the preparation of a protein conjugate of the formula



I-A

wherein R^1, R^4, m, n and the protein are as in claim 1.

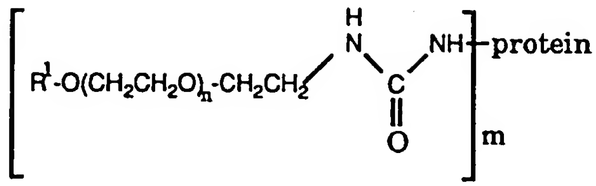
5. A process of any one of claims 1-3 for the preparation of a protein conjugate of the formula



I-B

wherein R^1, m, n and the protein are as in claim 1.

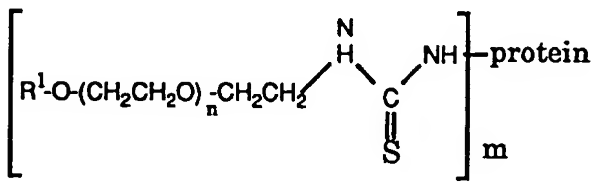
6. A process of any one of claims 1-3 for the preparation of a protein conjugate of the formula



I-C

wherein R^1, m, n and the protein are as in claim 1.

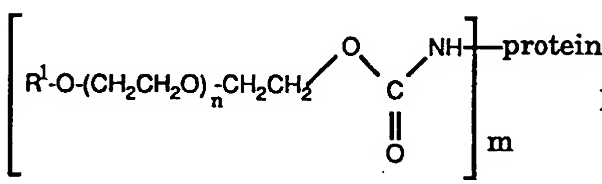
7. A process of any one of claims 1-3 for the preparation of a protein conjugate of the formula



I-D

wherein R^1, m, n and the protein are as in claim 1.

8. A process of any one of claims 1-3 for the preparation of a protein conjugate of the formula



I-E

wherein R^1, m, n and the protein are as in claim 1.

9. A process according to any one of claims 1-8 wherein R^1 is CH_3

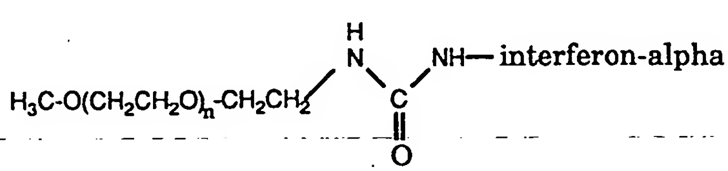
10. A process according to any one of claims 1-9 wherein the protein is interleukin-1.

11. A process according to any one of claims 1-9 wherein the protein is interleukin-1ra.

12. A process according to any one of claims 1-9 wherein the protein is interferon-alpha.

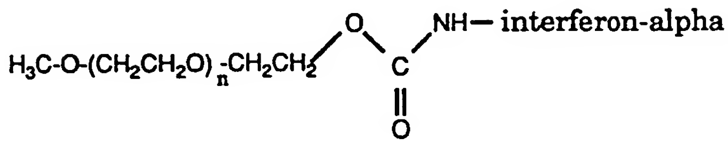
13. A process according to any one of claims 1-9 wherein the protein is interleukin-2.

14. A process of claim 1 for the preparation of a protein conjugate of the formula



wherein n is about 112.

15. A process of claim 1 for the preparation of a protein conjugate of the formula



wherein n is about 112.

16. A process according to claim 12 or claim 13 wherein the interferon-alpha is interferon-alpha A.

17. A process for the manufacture of a pharmaceutical composition which process comprises, bringing one or more protein conjugates of the formula 1 defined in any one of claims 1-16 and, if desired, one or more other therapeutically valuable substances into a suitable galenical dosage form.

18. Pharmaceutical compositions comprising a protein conjugate as claimed in any one of claims 1-16 and a therapeutically inert carrier.

19. Pharmaceutical compositions for the treatment or prophylaxis of immunomodulatory disorders such as neoplastic diseases or infectious diseases comprising a protein conjugate as claimed in any one of claims 1-16 and a therapeutically inert carrier.

20. The use of the protein conjugates according to any one of claims 1-16 in the treatment or prophylaxis of illnesses of others than human beings.

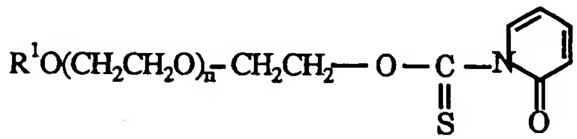
21. The use of the protein conjugates according to any one of claims 1-16 for the manufacture of medicaments for use in the treatment or prophylaxis of illnesses.

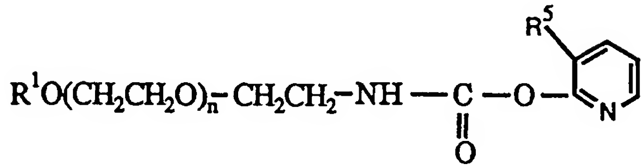
22. A protein conjugate as claimed in any one of claims 1-16 whenever prepared according to the process as claimed in claim 1 or by an obvious chemical equivalent thereof.

23. The invention substantially as hereinbefore described especially with reference to the foregoing Examples.

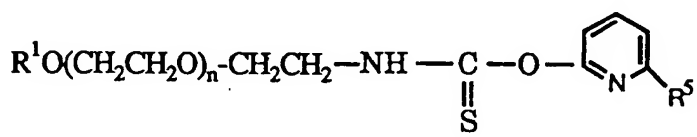
24. A compound selected from the group consisting of

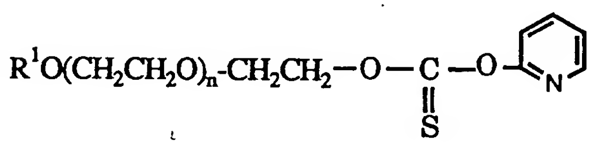
II-A $R^1O(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}N{=}C{=}N\text{-}R^4$

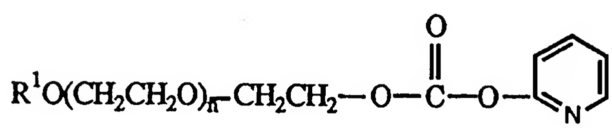
II-B 

II-C 

II-D $R^1O(CH_2CH_2O)_n\text{-}CH_2\text{-}CH_2\text{-}N{=}C{=}S$

II-E 

II-F 

II-G 

wherein R^1 is lower alkyl, R^4 is lower alkyl or cycloalkyl, R^5 is lower alkyl or H and n is any integer of from 1 to 1000.

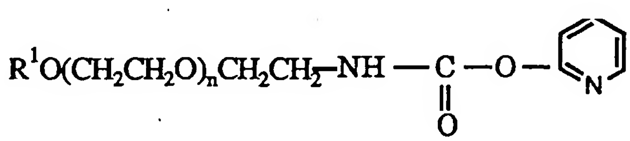
25. A compound of claim 24 wherein R^4 is cyclohexyl.

26. A compound of claim 24 wherein R^5 is H.

27. A compound of claim 24 wherein R^5 is CH_3.

28. A compound according to any one of claims 24-27 wherein R^1 is CH_3.

29. A compound with the formula



wherein R^1 is CH_3 and n is about 112.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number

EP 92 10 4729

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-0 304 311 (THE WELLCOME FOUNDATION LTD) * Whole document *	1-3,8,9,23,25-28	A 61 K 47/48
D,X	WO-A-9 007 938 (CETUS CORP.) * Page 7, line 17 - page 8, line 3; claims *	1-3,8-11,13,23,25-28	
D,Y		1,10-16	
X	WO-A-9 005 534 (GENENTECH, INC.) * Page 6, lines 6-26 *	1-3,8,9,23,25-28	
Y	WO-A-9 004 606 (ROYAL FREE HOSPITAL SCHOOL OF MEDICINE) * Pages 1-7 *	1,10-16	
X	WO-A-8 906 546 (CETUS CORP.) * Page 16, line 15 - page 22, line 28 *	1-3,8,9,23,25-28	

-/-

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

A 61 K
C 08 G

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely: 1-16,22,23,25-28

Claims searched incompletely: 17-21,24

Claims not searched:

Reason for the limitation of the search:

For economic reasons and in view of the examples of the application, the search has been restricted to the compounds of claims 17-22 in which the valve of n is 1, and greater than 10. See Guidelines for Examination in the European Patent Office, Part B, Chapter II, 7.

Place of search	Date of completion of the search	Examiner
THE HAGUE	13-07-1992	SITCH W.D.C.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 92 10 4729

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
P,X	WO-A-9 107 190 (ENZON, INC.) * Claims 1-41 * ---	1-3,8,9,23,25-28	
P,X	WO-A-9 108 229 (BASF AG) * Page 2, line 42 - page 7, line 25 * ---	1-3,6,8,9,23,25-28	
D,X	INT. ARCHS. ALLERGY APPL. IMMUN., vol. 66, 1981, pages 439-446, Basel, CH; T.P. KING et al.: "Immunologic properties of conjugates of ragweed antigen E with various alkoxypolyethylene glycols" * Page 439, abstract; page 440, figure 1 * ---	1-3,5,9,23,25-28	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
A	CHEMICAL ABSTRACTS, vol. 101, 1984, page 700, abstract no. 130594m, Columbus, Ohio, US; & HU-A-30 766 (F. SZEGO) 28-03-1984 * Abstract * -----	17,19-22	